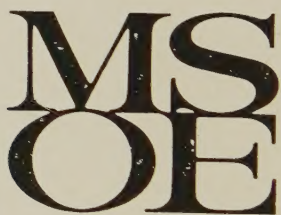


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AN INTRODUCTION TO
PRACTICAL BACTERIOLOGY

AN INTRODUCTION TO PRACTICAL BACTERIOLOGY

AS APPLIED TO MEDICINE AND
PUBLIC HEALTH

*A Guide to Bacteriological Laboratory Work
for Students and Practitioners of Medicine*

BY

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AN INTRODUCTION TO
PRACTICAL
BACTERIOLOGY

BY
J. H. ROBERTSON, F.R.S.E., F.R.S.S., F.R.S.M.
FELLOW OF THE ROYAL SOCIETY OF MEDICAL OFFICERS

LECTURER IN BACTERIOLOGY AND
PUBLIC HEALTH IN THE UNIVERSITY OF EDINBURGH

THIRD EDITION

WITH ILLUSTRATIONS BY
J. H. ROBERTSON, F.R.S.E., F.R.S.S., F.R.S.M.
FELLOW OF THE ROYAL SOCIETY OF MEDICAL OFFICERS

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PREFACE

THE scope of bacteriological study has widened so considerably in recent years that the under-graduate, and even the post-graduate worker, finds some difficulty in utilising the text-books for direct guidance in practical work. We have appreciated this from our own experience in teaching, and have therefore endeavoured to set forth as concisely as possible in book form the essential methods and data relating to practical bacteriology and bacteriological diagnosis.

The book has been prepared primarily for the use of students in the University of Edinburgh, and the arrangement of the subject matter is therefore in accordance with our own system of teaching. It represents only what its title indicates—an introduction and guide to practical bacteriology as studied by medical under-graduate and post-graduate students. It is in no way intended as an abbreviated text-book or an epitome of medical bacteriology, and does not include subjects dealt with in advanced classes or those usually studied apart from actual laboratory exercises.

The student is expected to use the book for guidance in a laboratory course in which the various apparatus, microscopic appearances, cultural characters, biochemical and serological reactions are adequately demonstrated, and, with the exception of a few diagrams required to facilitate description, illustrations are omitted.

We have attempted to emphasise the more important methods used in routine bacteriological diagnosis,

recommending those we have found most serviceable in our own experience. Due attention has also been paid to the diagnosis of tropical infections. It is hoped that the text may prove of value to medical workers undertaking certain bacteriological methods for the first time and also to practitioners when submitting specimens for diagnostic tests.

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January, 1925.

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I

THE GENERAL BIOLOGY OF MICRO-ORGANISMS

THE BIOLOGICAL DIVISIONS REPRESENTED BY THE PATHOGENIC MICROBES

BACTERIOLOGY, as applied to medicine, embraces the study of those micro-organisms which are pathogenic to, or commensals of, man. They may be broadly classified in the following biological divisions ¹:—(1) the bacteria or schizomycetes; (2) the hyphomycetes (or true fungi) and the blastomycetes (yeasts); (3) the protozoa.

Certain pathogenic organisms, however, are either so minute or so plastic that they can pass through an earthenware filter which is impervious to the recognised bacteria, and are designated “filterable viruses.” The majority still remain biologically undefined.

The differential characterisation of these biological divisions is as follows:—

BACTERIA.—Very minute, simple, unicellular organisms, multiplying with great rapidity and reproducing usually by transverse fission; devoid of chlorophyll and exhibiting no morphological nucleus; in certain species developing a resting phase in the form of “spores” or “gonidia.”

HYPHOMYCETES.—Branching filaments (hyphae)

¹ The biological nomenclature and classification used in this work do not follow strictly the systems at present accepted in biological literature, but represent one that has been found of most service in introducing medical students to practical bacteriological work. The classification of micro-organisms is in many respects a matter of controversy, and in medical bacteriology a simple scheme of classification with an expressive nomenclature amply fulfils the practical needs of the subject.

interlacing and forming a meshwork (mycelium); more highly organised than the bacteria, often septate and multicellular, and reproducing usually by means of spores developed in "fruiting organs."

BLASTOMYCETES.—Round, oval or elongated cells, generally larger than bacteria and multiplying by "budding"; in some species multiple "endospores" formed, and in some, elongation into hyphae noted.

PROTOZOA.—Generally regarded as the lowest forms of animal life; minute unicellular organisms with the protoplasm differentiated into nucleus and cytoplasm; reproducing by fission and spore-formation, and often exhibiting a definite life-cycle with sexual and asexual phases.

The bacteria and the filterable viruses play the most important part in the causation of human infective disease. Protozoal infections are most prevalent in tropical and subtropical countries.

THE BACTERIA

For practical work some system of biological classification is necessary. It is generally sufficient to classify the bacteria in the first instance into the following main subdivisions:—

Higher Bacteria (or *Trichobacteria*)

Elongated sheathed filaments, often showing true or false branching; cells interdependent, some specialised for reproduction.

Lower Bacteria (or *Eubacteria*)

Simple unicellular structures never in the form of sheathed filaments; each cell equivalent; many species motile, usually due to their possessing flagella.

Genera of Medical Interest

1. *Leptothrix*—unbranched.

Main Morphological Classes

1. *Cocci*—globose in shape.
2. *Bacilli*—straight (or

2. *Streptothrix* — shows true branching, and filaments interlace to form a mycelium. only slightly curved) rod-shaped organisms.
3. *Vibrios* and *spirilla*— definitely curved rods, or spirals, which are non-flexuous.
4. *Spirochaetes*—filaments showing spirals or undulations; generally flexuous; multiplying by longitudinal as well as by transverse fission.

MORPHOLOGICAL STUDY OF THE BACTERIA

UNSTAINED PREPARATIONS. — The morphology of the bacteria can be studied by examining them first in the unstained condition suspended in some fluid material. In this way their general shape can be observed and motility determined, but their refractility is not marked, and in the case of delicate organisms, such as the *spirochaetes*, dark-ground illumination (*vide* p. 42) is necessary for their demonstration.

STAINED PREPARATIONS. — The examination of stained preparations is usually an essential routine procedure. For this purpose various aniline dyes are employed, often along with a mordant. For the demonstration of *spirochaetes* “negative staining” has been used—*i.e.* where the organism is mixed with some substance such as Indian ink, which, in film preparations, yields a dark background, while the organism stands out white and unstained. Silver impregnation methods (*vide* p. 115) are also utilised for the routine demonstration of *spirochaetes*.

STAINING REACTIONS. — The staining reactions of the bacteria are of the greatest importance both in morphological studies and for differentiation and identification. Thus, all the bacteria can be divided into two categories by the so-called Gram’s staining reaction (*vide* p. 97)—*i.e.* according to whether they

resist decolorisation with aniline oil or alcohol after staining with gentian violet and subsequent treatment with iodine. Those retaining the dye are designated "Gram-positive"; those that become decolorised, "Gram-negative."

Some bacteria, when stained, resist decolorisation with acid, and are spoken of as "acid-fast"—*e.g.* the tubercle bacillus (*vide* p. 102).

Certain organisms do not stain uniformly and this may be a characteristic feature. Thus the diphtheria bacillus shows "beading" when stained with methylene blue. The plague bacillus displays "bipolar staining," the ends being more deeply coloured than the centre.

Another staining reaction that is characteristic of certain species—*e.g.* *B. diphtheriae*—is the appearance of metachromatic granules—*i.e.* where certain granules are demonstrated in the bacterial protoplasm, which can be stained with one stain while the rest of the protoplasm stains with a dye of different colour (*vide* p. 105).

PLEOMORPHISM AND INVOLUTION.—It must be remembered that, in artificial culture, bacteria may show considerable variation in shape and size (pleomorphism), and may also exhibit degeneration or involution forms which are different morphologically from the normal cell.

MOTILITY.—The motility of bacteria observed in a fluid medium is generally due to delicate prolongations of the protoplasm (flagella) which act as locomotory organs. These are not seen in unstained preparations and can only be demonstrated by special staining methods (*vide* p. 109). Brownian movement must not be confused with true motility. In the latter case the organism definitely changes its position in the microscope field (*vide* p. 42).

Flagella may be "terminal"—*i.e.* at one or both ends of the bacterium, and single or multiple. They may be distributed all round the organism, and this arrangement is described as "peritrichous."

Among the spirochaetes motility is generally due to contractions of the cell body, but in certain species flagella also contribute to movement.

BACTERIAL SPORES.—Some species develop a highly resistant resting-phase, or spore, by which the individual survives unfavourable external conditions. The spore is not a reproductive structure. Only one spore is developed by each vegetative form. This structure appears in the cell protoplasm (“endogenous”) and increases in size, appearing as a round, oval or elongated body, which may be situated in the centre of the bacterium (“central”), at the end (“terminal”), or between the centre and end (“subterminal”). The relative size of the spore varies with different species. Spores can withstand all injurious chemical and physical influences better than the vegetative forms, and owe their resistance to a dense outer protective membrane. Under favourable external conditions the membrane ruptures and the vegetative form is restored.

The spore is not stained by the ordinary methods and appears as a clear, unstained portion of the bacterial protoplasm. Special methods can be used for the differential staining of spores (*vide* p. 107).

BACTERIAL CAPSULES.—Certain bacteria possess a relatively thick outer capsule and are described as “capsulated.” By ordinary methods of staining this capsule may appear as an unstained zone round the organism. Special methods are available for capsule staining (*vide* p. 107).

THE BACTERIAL PROTOPLASM.—With regard to the structure of the bacterial protoplasm, no morphological nucleus can be demonstrated, and if bacteria contain nuclear material, we must regard it as diffused throughout the cytoplasm. It is of interest that the cell protoplasm exhibits an affinity for those basic aniline dyes which are used as nuclear stains.

MULTIPLICATION AMONG THE BACTERIA.—Multiplication takes place usually by transverse fission. The cell elongates, and a constriction develops transversely which

ultimately divides the original individual into two new cells. Division may occur with great rapidity—*e.g.* every half-hour—so that one individual may soon reproduce several millions of new cells. Among the spirochaetes both longitudinal and transverse fission are noted.

In the higher bacteria transverse division occurs. Certain filaments also divide up at their free end into a number of oval “gonidia,” which are set free, and each of these, besides representing a resting-phase, may develop a new colony.

COCCHI

Cocci are classified morphologically as follows :—

Staphylococci.—The individuals are arranged in clusters, due to the irregularity of the planes of successive divisions.

Streptococci.—The cocci are grouped in chains, the planes of successive fission being parallel.

Tetrads.—Plates of four (or multiples of four) cocci, division occurring successively in two planes at right angles.

Sarcinae.—Packets of eight (or multiples of eight) cocci, division occurring successively in three planes at right angles.

Diplococci.—The cells tend to be arranged uniformly in pairs.

The different cocci are relatively uniform in size, 1μ being the average approximate diameter. Some species are capsulated. Shape varies with species, being spherical, oval, lanceolate, or bean-shaped. Gram-staining is an important criterion in identification.

BACILLI

Morphological features that are of importance in the study and identification of these organisms are :

Size—some being relatively large—*e.g.* *B. anthracis* (*vide* p. 194), others small—*e.g.* *B. influenzae* (*vide* p. 234).

Shape—rectangular—*e.g.* *B. anthracis*; oval (*coccobacilli*)—*e.g.* *B. pestis*.

Arrangement—in pairs—*e.g.* *pneumobacillus*; in chains—*e.g.* *B. anthracis*.

Occurrence of a capsule.

Motility, flagella and their arrangement.

Spores and their position.

Gram-staining.

Acid-fastness—*e.g.* *tubercle bacillus*.

Staining of cytoplasm—*e.g.* “beading,” bipolar staining, metachromatic granules (*vide supra*).

VIBRIOS AND SPIRILLA

“Comma”-shaped forms, “S” forms and spirals are characteristic. Most species are very actively motile (*e.g.* “darting” motility) and the flagella are terminal. They are usually Gram-negative.

SPIROCHAETES

The pathogenic varieties can be broadly classified into the following genera :—

(1) *Spironema*

(2) *Leptospira*

the latter being differentiated by its numerous and very fine coils observable by the dark-ground illumination method.

Some spirochaetes are relatively large, refractile and easily stained by ordinary methods; others are delicate, feebly refractile and difficult to stain—*e.g.* *Spironema pallidum*. The coils may be regular corkscrew-like spirals¹ or only irregular curves. Movement is undulating or rotatory. It has been noted in certain pathogenic species that granules are developed in the protoplasm and extruded from the cell, and these are regarded as a resting-phase, being analogous to the bacterial spore.

¹ The generic term “*treponema*” has also been applied to those spirochaetes with regular corkscrew-like spirals—*e.g.* the spirochaete of syphilis (*vide p. 256*).

PHYSIOLOGY OF THE BACTERIA

The physiology and biochemistry of bacteria can be readily studied by observations made with "cultures" growing in the laboratory in a nutrient medium.

Bacteria are subject, as regards their growth and vitality, to various external influences—*e.g.* atmosphere, temperature and moisture of their environment, light, chemicals, etc.

ATMOSPHERE.—Some species require an abundant supply of *free* oxygen for their growth and are described as "obligatory aerobes." Others will not grow in the presence of *free* oxygen ("obligatory anaerobes"). The majority of pathogens are, however, indifferent in this respect, and will flourish in the absence of oxygen, though they prefer an oxygen-containing atmosphere ("facultative anaerobes") (*vide* p. 85).

TEMPERATURE.—(a) *Influence on Growth.*—For each species there is a definite temperature range within which growth takes place. The limits are the "maximum" and "minimum" temperatures, and an intermediate "optimum" temperature can usually be estimated at which the best growth occurs. The optimum temperature of a bacterium is approximately that of its natural habitat—*e.g.* 37.5°C. in the case of the pathogens of man. Some organisms have a wide temperature range, others are more restricted. Beyond this range viability is not necessarily interfered with.

(b) *Influence on Viability.*—Heat is an important agent in the artificial destruction of micro-organisms. Non-sporing bacteria cannot withstand temperatures above 57°C. for any length of time.

The "*thermal death point*" is the lowest temperature (above the maximum for growth) which will kill a particular organism in ten minutes.

Bacteria are more susceptible to moist than dry heat. Spores are much more resistant to heat than vegetative bacteria. Further data on this subject are given in regard to sterilisation (p. 47).

Some species die if kept at 0°C., but others may survive even lower temperatures.

MOISTURE.—Four-fifths by weight of the bacterial cell consists of water, and, as in the case of other organisms, moisture is necessary for growth. Drying is generally injurious, except to spores which can resist this influence for long periods.

LIGHT.—The optimum condition for growth and viability is darkness. Ultra-violet rays are markedly bactericidal.

INFLUENCE OF CHEMICALS.—Various inorganic and organic chemicals, acting as protoplasmic poisons, are either inhibitory to growth or bactericidal, depending on the concentration brought into contact with the particular organism.

FOOD SUPPLY.—The growth of bacteria is, of course, dependent on an adequate supply of suitable food material. This varies with the natural adaptations of different species. Thus, as a general rule, in the artificial culture of the pathogens the medium should approximate to the composition of the tissues and body fluids (*vide* p. 54). The H-ion concentration of the medium is also an essential factor in influencing growth (*vide* p. 60).

MUTUAL INFLUENCES.—Different bacteria may flourish well together, the presence of one species favouring the growth of another—*symbiosis*. The reverse effect may also be observed—*antibiosis*—where one species is antagonistic to another.

BACTERIAL ENZYMES.—In the metabolism of bacteria and in the biochemistry of bacterial action enzymes play an essential part. One of the great functions of bacteria in nature is to produce chemical decomposition of complex organic substances—*e.g.* proteins and carbohydrates—by means of their enzymes. Among the pathogens fermentative properties are important features in the identification of certain species.

Some bacteria, including certain pathogens, are **CHROMOGENIC**, and produce characteristic pigments.

BACTERIAL TOXINS.—These are defined as the products

of bacteria which are injurious to the tissues and in virtue of which disease processes result from bacterial infection. They have not been isolated as chemically pure substances, but are generally regarded as being of protein nature. They are classified as :

1. Extracellular or exotoxins, which diffuse readily from the bacteria into the surrounding medium.
2. Intracellular or endotoxins, which are retained within the cells until the bacteria die and disintegrate.

The majority of the pathogens produce endotoxin only, but certain develop powerful exotoxins—*e.g.* *B. diphtheriae*, *B. tetani*, *B. dysenteriae* (Shiga), *B. botulinus*.

The exotoxins are specific in their action on particular tissues. They can be prepared by growing the bacteria in fluid culture and filtering through a porcelain filter—the filtrate contains the toxin (*vide* pp. 52, 181).

The endotoxins are less specific in their action. They can be readily tested by injecting dead bacteria into animals.

Special toxins have been demonstrated with particular effects—*e.g.* haemolysins (producing lysis of blood), leucocidins (destructive to leucocytes).

VIRULENCE OF MICRO-ORGANISMS.—Virulence has generally been regarded as an important property of micro-organisms in relation to their pathogenesis, and is defined as the capacity to multiply in the tissues and produce toxic effects. Virulence is estimated by the *minimum lethal dose*—*i.e.* the smallest dose of the organism (*e.g.* in the form of a culture) which will kill a particular species of animal.

The virulence of an organism can be “exalted” or “attenuated” artificially.

Exaltation of virulence for a particular animal species is produced by passing the strain through a series of individuals of the same species.

Attenuation usually results when organisms are cultivated artificially for some time, so that the stock laboratory cultures are usually of low virulence, as compared with recently isolated strains.

SYSTEM OF BIOLOGICAL CLASSIFICATION AND IDENTIFICATION OF THE BACTERIA

1. THE MORPHOLOGY, TOGETHER WITH THE STAINING REACTIONS, OF INDIVIDUAL CELLS generally serves as a preliminary criterion, particularly for placing an unknown organism in its appropriate biological group. In medical bacteriology the microscopic characters of certain organisms in pathological specimens may be sufficient for diagnostic identification—*e.g.* tubercle bacilli in sputum. Morphology among the bacteria usually fails, however, to differentiate allied organisms—*e.g.* meningococcus, gonococcus, *Micrococcus catarrhalis*.

2. CULTURAL CHARACTERS, OR THE MORPHOLOGY OF GROWTHS ON ARTIFICIAL MEDIUM—*e.g.* the appearances of “colonies” to the naked eye or with certain magnifications. This criterion is important in identification, but may also be insufficient to differentiate species—*e.g.* coli-typhoid group.

3. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERS—*e.g.* the fermentation of various carbohydrates. Species which cannot be distinguished by morphology and cultural characters may exhibit marked differences in their biochemical reactions—*e.g.* coli-typhoid group. Different species may, however, resemble one another closely in fermentative tests—*e.g.* *B. paratyphosus* B, and *B. enteritidis* Gaertner.

4. ANIMAL EXPERIMENT.—In the case of pathogens that are virulent to, and produce characteristic lesions in, laboratory animals, the inoculation test provides a certain method of identification—*e.g.* *B. tuberculosis*.

5. SEROLOGICAL TESTS.—In bacteriology, species and types can often be accurately identified by specific “antigen-antibody reactions.” These depend on the fact that the serum of an animal immunised against a micro-organism contains specific antibodies (for the homologous species) which react in a characteristic manner with the particular organism (antigen) (*vide* p. 26). Such antisera, for example, may agglutinate

or clump the homologous organism in test-tube experiment, and this effect can easily be observed even with the naked eye.

It may also be noted here that the serum of a person suffering from a bacterial infection may agglutinate the particular organism, and in this way the agglutination reaction can be used for diagnostic purposes.

FUNGI; PROTOZOA

The general morphology and physiology of the fungi and the protozoa need hardly be dealt with here. The biological aspects of the types that are of special importance in practical bacteriology as applied to medicine will be referred to later in the consideration of the particular organisms.

FILTERABLE VIRUSES

Little is yet known regarding the biology of these viruses. It is usually assumed that they represent living organisms, owing to the fact that they can be propagated through a series of animals. The majority, though demonstrable as viruses by the experimental method, have not been observed microscopically, and the term "ultramicroscopic" has also been applied to them. Exceedingly minute filterable organisms, however, have been demonstrated microscopically and even cultivated artificially—*e.g.* *Bacterium pneumosintes* in epidemic influenza (*vide* p. 284).

These viruses, like other micro-organisms, are easily destroyed by injurious physical and chemical agencies. As compared with the bacteria, they possess a high degree of resistance to glycerol.

Their demonstration and identification depend on the experimental production of a characteristic pathological condition in animals (or man) by means of filtrates after subjecting material from the particular disease (*e.g.* nasal washings, serum) to filtration through an earthenware filter (*vide* p. 278).

II

IMMUNITY IN RELATION TO PRACTICAL BACTERIOLOGY

THE subject of immunity is intimately related to practical bacteriology, and immunological principles underlie certain bacteriological methods.

The term "immunity" signifies the power of the animal body to resist infection with parasitic microbes or the injurious effects of their products.

Immunity may be *acquired*, as in the natural recovery from infection, and is due to the development, during the illness, of new and specific resisting powers against the causal organism or its toxin.

An acquired immunity may also be developed artificially by inoculating an animal with a virus so altered that it is incapable of reproducing the actual disease though still able to bring about an immunity reaction. Such immunity is described as an *active artificial immunity*.

The blood serum of an actively immunised animal introduced into the body of a non-immune animal renders the latter temporarily immune, and this is termed *passive immunisation*.

The most frequent methods of producing an active artificial immunity are :

1. Introduction of heat-killed cultures (vaccines) in successive and increasing doses. This system is applied in the prophylaxis of certain infections (*e.g.* anti-typhoid vaccination), in therapeutic immunisation (*e.g.* treatment of chronic infections with vaccines), and also in the preparation of antibacterial sera in animals.
2. Introduction of toxins in successive and increasing

doses, so that each dose is devoid of harmful effect. This is exemplified in the preparation of diphtheria and tetanus antitoxins.

The serum of an actively immunised animal is designated an *immune serum* or *antiserum*, and owes its action to specific *antibodies* or *immune bodies* which act adversely on the homologous organism or neutralise its toxins.

The special principle in the organism or its toxin which incites antibody production is described as an *antigen*.

A serum containing antibodies antagonistic to the particular bacterium is spoken of as an *antibacterial serum*; one which contains antibodies that neutralise toxin, as an *antitoxic serum*.

It has to be noted that, apart from antibacterial and antitoxic immunity, other cells, and in fact all protein substances, may act as antigens and incite specific antibody production—*e.g.* red blood corpuscles, blood serum, ferments, animal venoms, etc.

Thus the red cells of one animal injected into another animal of different species leads to the development of antibodies in the serum of the latter which, under certain conditions, can effect lysis of the blood (*haemolysis*) of the former. This antiserum is described as a *haemolytic antiserum*, and the antibody as a *haemolysin*.

As a general rule the antigen must be injected parenterally to produce immunity.

Usually an animal can only be immunised against an antigen foreign to its own tissues.

Specificity for the particular antigen is one of the pronounced characters of antibodies, and is usually for the biological species, though in certain cases it may be more restricted.

It must be remembered that, in some instances, antibodies of practical importance may have no aetiological significance. Thus, in typhus fever an antibody may occur in the serum which is specific for *B. proteus*—though this organism has no aetiological relationship to the disease.

An antitoxic serum is produced by immunising an animal with exotoxin. For example, "diphtheria antitoxin" is the serum of a horse which has been immunised with graded doses of diphtheria toxin. When appropriate amounts of toxin and antitoxin are mixed together the mixture is non-toxic. This process of neutralisation is complex and need not be dealt with here. It occurs both *in vivo* and *in vitro*.

Antibacterial sera are generally produced by immunising with the actual bacteria or their endotoxin.

These sera may exhibit the following properties:—

1. *Bacteriolytic* or *bactericidal*—*i.e.* directly destroying the bacteria.
2. *Opsonic* or *bacteriotropic*—*i.e.* rendering the bacteria susceptible to phagocytosis.
3. *Agglutinating* or clumping the bacteria.
4. *Precipitating* or producing a precipitate with the soluble products of the bacteria.
5. *Complement-deviating*—*i.e.* along with the antigen "absorbing," "fixing" or "deviating" complement (*vide infra*).

These effects may be demonstrated *in vitro*. They are all relatively specific for the particular organism.

The serum of a person or animal infected with pathogenic bacteria may also exhibit similar properties and constitutes an antibacterial serum.

It has to be noted that normal serum possesses non-specific bactericidal and bacteriotropic effects. These are relatively weak as compared with the corresponding specific effects. The bactericidal action of normal serum is due to a non-specific, thermolabile (at 55°C.) constituent called *alexin* or *bactericidal complement*. The opsonic effect of normal serum is also due to a non-specific, thermolabile principle, the *normal opsonin*.

The bactericidal action of an immune serum is produced by a specific thermostable antibody (*bacteriolysin*) acting along with the normal complement. It has been supposed that the antibody acts by bringing more complement into combination with the bacteria than in the case of normal sera, and represents a

combining link or *amboceptor* between the bacteria and the complement.

Thus :

Bacteria + {Specific
antibody} + Complement = Bacteriolysis.

Bacteria + {Specific
antibody} = No effect.

Haemolysis by a haemolytic antiserum is analogous to bacteriolysis—*i.e.* it is due to a specific thermostable antibody acting along with the normal complement.

Thus :

Red Cells + {Specific
antibody} + Complement = Haemolysis.

Red Cells + {Specific
antibody} = No effect.

Red Cells + Complement = No effect.

A suspension of red corpuscles in isotonic salt solution *plus* the homologous antiserum which has been heated at 55° C. to remove complement (*i.e.* red cells + specific antibody only), serves as a test for the presence or absence of complement—*e.g.* in complement-deviation tests, and is spoken of as a *haemolytic system* (*vide* p. 140).

The increased opsonic action of an antibacterial serum is due to a specific thermostable antibody (*immune opsonin*).

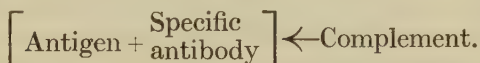
Agglutination is attributed to a specific antibody designated *agglutinin*. The agglutinin does not affect the vitality of the bacteria. It also clumps dead bacteria in the same way as the living organisms. It is relatively thermostable. Salts are necessary for its action and agglutination tests are usually carried out in a medium of normal saline. These tests are applied in diagnostic work, and for the identification of species and types (*vide* p. 134).

The precipitating effect of an antibacterial serum is regarded as due to a separate antibody called *precipitin*.

Complement plays an essential part in the bacteriolytic action of an immune serum, and is absorbed by

the bacteria *plus* bacteriolytic immune body. Apart from bacteriolysin, an immune serum may also contain antibodies which, along with antigen, absorb or deviate complement; and a separate *complement-deviating antibody* is therefore spoken of.

Thus :



To test this effect the haemolytic system is used as an indicator : if complement has been deviated, on adding the haemolytic system no haemolysis will occur (*vide* p. 139).

Complement-deviation tests are employed in diagnosis, and occasionally for the identification of species in the same way as the agglutination reaction.

III

THE USE OF THE MICROSCOPE IN BACTERIOLOGY

MICROSCOPY is of primary importance in Bacteriology, and a suitable microscope and a knowledge of its use are essential to those engaged in bacteriological work. To obtain satisfactory results, the microscope should be used under the best possible conditions, and the worker must know the uses and limitations of the instrument.

The component parts of the microscope are as follows:—

The *eye-piece* fits into a graduated *draw-tube*, which in turn slides in the *tube*. The lower end of the tube is furnished with a *revolving nose-piece* (preferably “triple”), into which are screwed three *objectives*. The focal lengths of these objectives should be: 16 mm., or $\frac{2}{3}$ in. (low power); 4 mm., or $\frac{1}{6}$ in. (high power); and 2 mm., or $\frac{1}{12}$ in. (oil immersion) (*vide infra*). The tube and objectives are moved up and down by means of a rack-and-pinion movement termed the *coarse adjustment*, while the fine movements necessary for accurate focussing are performed with the *fine adjustment*. The tube and adjustments are supported by an *upright* which is connected to the *foot* by means of a *hinged joint*. Attached to the upright near the joint is a platform called the *stage*, which may have a plain surface or may be fitted with a *mechanical stage*. Below the stage is the *substage*, which should be furnished with a means of raising or lowering—usually a rack and pinion or a spiral screw. The substage is fitted with a *substage condenser*, attached to which is the *iris diaphragm*. Fitted to the end of the *tail-piece* is a *mirror*, mounted on a gimbal fitting. The plane side of the

mirror is employed when a condenser is used; the concave side is used only in the absence of a condenser, and its focal length is such that light comes to a focus on the object examined.

The draw-tube should be adjusted to the tube length of the objective, which is usually 160 mm., but as the revolving nose-piece has a length of 18 mm., the draw-tube should be extended only to the 142 mm. mark. One well-known microscope has the objectives corrected for a 170 mm. tube length. It is essential that an objective should be used at its proper tube length, particularly the apochromatic objectives (*vide infra*), if the maximum resolution is to be obtained.

The manner in which the fine adjustment works varies according to the make of the microscope, for different manufacturers have their own particular type of mechanism. The older forms were actuated by means of a milled head mounted on a pillar behind the coarse adjustment, which turned a screw with a fine thread. Newer models have a milled head parallel to the coarse adjustment. The movements of the milled head raise or lower the tube in the same direction as the coarse adjustment. The milled head is graduated in $\frac{1}{100}$ ths, and one division corresponds usually to a movement of the tube of 0.001 mm. The manner of securing a fine movement is by a system of either levers, cams or cogwheels.

The stage should be large enough to take a $3\frac{1}{2}$ in. Petri dish, and if plain, should be fitted with two clips. A mechanical stage is of great advantage, and is particularly useful where a large area of a microscopic preparation has to be searched, as in the examination of films of sputum for tubercle bacilli, or of blood for malaria parasites. An attachable mechanical stage works satisfactorily and can be obtained for almost any model. The "built-in" mechanical stage is more costly, but is to be recommended for extensive routine bacteriological work, as it is steadier and not likely to get out of order.

The substage is an important part of the microscope

and one to which, unfortunately, little attention is paid. The mechanism of raising the substage should be rigid and free from lateral movement. Where critical work has to be done with highly corrected objectives and condenser, centering screws should be fitted. The condenser, which is used for focussing light on the object to be examined, is usually of the two-lens Abbe type; but if apochromatic or semi-apochromatic objectives are used, a condenser of similar optical correction must be employed. The iris diaphragm is an important essential of the substage, as it controls the amount of light passing into the condenser.

OBJECTIVES AND EYE-PIECES

For general purposes, ordinary achromatic objectives are quite satisfactory, and are admirable for routine work and students' use. The quality of the present-day objective is extremely good, and for ordinary work the purchase of the more expensive types is not recommended. The most useful objectives are $\frac{3}{8}$ -in. or 16 mm., $\frac{1}{8}$ -in. or 4 mm., and the $\frac{1}{12}$ -in. (thus designated, but actually $\frac{1}{14}$ -in.) oil immersion. These should be used in conjunction with a 10 magnification ($10\times$) eye-piece. A $5\times$ eye-piece is often supplied, and is sometimes employed for searching when a larger field is desired without altering the objective. It is not practicable to use the ordinary (Huyghens) eye-piece above $12\times$, and even this magnification gives some distortion and haziness of outline.

MAGNIFICATION

The objective works at a distance from the object somewhat less than its focal length. A real, inverted and enlarged image is formed in the upper part of the tube. This real image is further magnified by the eye-piece, and the total magnification is the product of the separate magnifications of the objective and of the eye-piece. The objective acts as a convex lens and the magnification is easily calculated. The ratio of the size

of the image *to* the size of the object—*i.e.* the magnification—is the same as the ratio of the distance of the image from the objective *to* the distance of the object from the objective. In the microscope the distance of the image from the objective is constant—*i.e.* the tube length (160 mm.); and the distance between the objective and object is adjusted by means of the coarse adjustment—that is, the objective is focussed on the object. Suppose an object is examined with a 16 mm. ($\frac{2}{3}$ -in.) objective and a $10\times$ eye-piece; the size of the image produced by the objective alone depends on the ratio of the tube length to the focal length of the objective—*i.e.* 160 mm. *to* 16 mm. (ten times); this real image is now magnified ten times by the $10\times$ eye-piece, making a total magnification of 100 diameters. If a 4 mm. ($\frac{1}{6}$ -in.) objective is employed, the distance of the image is the same (160 mm.), but the distance between objective and object is only 4 mm., hence the initial magnification of the objective is 160 *to* 4—*i.e.* 40. This is further magnified by the $10\times$ eye-piece to 400 diameters. Similarly a 2 mm. objective has an initial magnification of 80, and when used in conjunction with a $10\times$ eye-piece gives a total magnification of 800 diameters.

Note.—The $\frac{1}{2}$ -in. objective has, in reality, a shorter focal length than that by which it is designated, and, when used in conjunction with a $10\times$ eye-piece, gives a magnification of approximately 1000 diameters. This is the magnification usually employed for bacteriological work.

It is thus seen that the magnification (M) varies inversely as the focal length (F) of the objective, the shorter the focal length the greater the magnification.

To find the magnification of any system of objective and eye-piece, divide the tube length employed by the focal length of the objective; multiply this figure by the magnification of the eye-piece and the total magnification is obtained.

The oil-immersion lens works very close to the cover-slip, and the intervening space between objective and cover-slip is filled with cedar-wood immersion oil.

The reason for this is that when a ray of light emerges from a dense medium (glass) into a rare medium (air), it is refracted outwards (see diagram—A B C D). As the brightness of the image depends upon the light entering the objective and the resolution (*vide infra*) depends

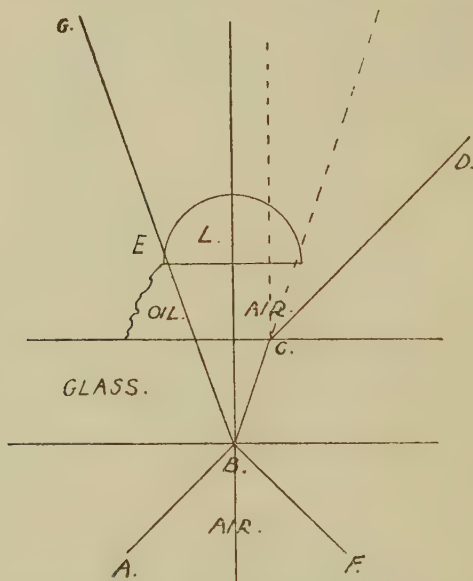


Diagram showing the paths of rays through (1) a dry lens (on right), and (2) an oil-immersion lens (on left) (*after Spitta*)

Note the refraction of the oblique ray A B C D in passing from the glass slide to air, as compared with the ray F B E G. L is the front lens of the objective

on the effective aperture, this refraction of light diminishes not only the brightness but the clearness of the image. If, however, the space between objective and image is occupied by immersion oil, which has the same refractive index as glass (1.5), the rays of light do not undergo refraction and pass into the objective (see diagram—F B E G.).

NUMERICAL APERTURE

Objectives are rated not only by their focal length but also by their *Numerical Aperture* (N.A.). The numerical aperture may be simply defined as the ratio of the diameter of the lens to the focal length.¹ It is expressed mathematically as follows:—

$$\text{N.A.} = n \sin U$$

where n is the refractive index of the medium between object and objective (air, 1.0; cedar-wood immersion oil, 1.515), and $2U$ the *angle of aperture*—i.e. the angle

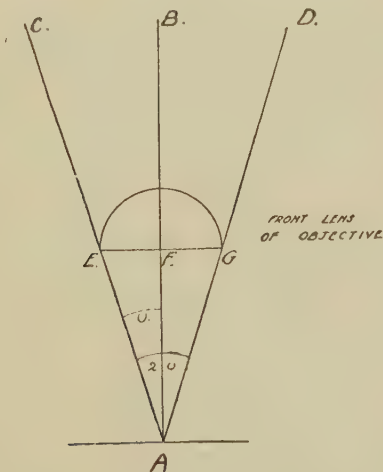


Diagram to illustrate numerical aperture

formed by the two extreme rays of light, which, starting from the centre point of the object, reach the eye of the observer.

¹ The numerical aperture has been expressed in this manner to simplify description, but this is true only for objectives of long focal length, where EA is approximately equal to FA. With short-focus lenses of high N.A. this definition is not correct. The length EA, on which the N.A. really depends, is then very much greater than the distance of the objective from the slide (FA).

That is $\text{DAC} = 2U$

$\text{BAC} = U$

$$\text{Sin } U = \frac{\text{EF}}{\text{EA}}$$

It is thus seen that the numerical aperture, other things being equal, depends on EF, which is half the diameter of the lens. Lenses, therefore, may have equal focal lengths, but different numerical apertures depending on the diameter of the lens. It is to be noted, however, that the numerical aperture is not measured directly by the diameter of the lens, but by the ratio of that diameter to the focal length.

The theoretical limit of the angle DAC is 180° —*i.e.* when the objective is actually on the object—and therefore the theoretical limit of U is 90° . The greatest possible N.A. of a dry lens can be only 1, since the refractive index of air = 1, and $\text{Sin } 90^\circ = 1$. Actually the highest practical N.A. of a dry lens is 0.95. On the other hand, the introduction of oil between the objective and object gives n a value of 1.5. The highest possible value, therefore, of $n \text{ Sin } U$ for an oil-immersion objective is $1.5 \times \text{Sin } 90^\circ$ —*i.e.* 1.5. In practice, however, the highest N.A. of an oil-immersion objective (attained in an apochromatic) is 1.40. The ordinary $\frac{1}{12}$ -in. objective for bacteriological purposes should have an N.A. of 1.30. The essential qualities of an objective depend on its numerical aperture, and these are :

- (1) *brightness of image*, which, other things being equal, varies as the *square* of the N.A. ;
- (2) *resolving power* and *defining power*, which vary directly as the N.A.

The *depth of focus*, while not dependent entirely on the N.A., varies in inverse proportion to it.

It is thus apparent why oil-immersion objectives give such good results—the N.A. being increased by the high refractive index of the oil. It may be said that in the case of two lenses of equal focal length the one with the higher N.A. is the better lens and is to be preferred. The 16 mm. or $\frac{2}{3}$ -in. objective should have

a minimum N.A. of 0.28; the 4 mm. or $\frac{1}{6}$ -in. a minimum N.A. of 0.65; while the $\frac{1}{2}$ -in. oil-immersion should have an N.A. of not less than 1.30.

The resolving power of a lens—that is, its capacity to separate two adjacent points (as apart from magnifying power)—varies directly as the N.A. The limit of resolution is attained when the magnification reaches 1450 diameters, and objects smaller than half the wave length of the light employed cannot be resolved—that is, objects less than 0.0001 mm. ($0.1\ \mu$) cannot be seen.

DEFINITION

This is the capacity of the objective to render the outline of an object distinct, and depends on the elimination of “spherical” and “chromatic” aberration.

Spherical aberration is caused by the periphery of the lens refracting more than the central portion. The peripheral rays, therefore, focus on the axis at a shorter distance from the lens than the central ones, with the result that the image is distorted.

Chromatic aberration is caused by the ray of white light being dispersed into its component colours as it is refracted through the lens, a spectrum being formed. The blue rays are refracted more, and come to a focus nearer the lens than the red rays. The different components do not come to the same focus and hence cannot blend to form white light. As a result, the image is fringed with colours and the outline is hazy.

Both chromatic and spherical aberration may be corrected by the combination of lenses of different dispersive power, convergent convex lenses of crown glass having low dispersive power, and divergent concave lenses of flint glass having high dispersive power. By this means two of the spectrum colours are combined and the ordinary achromatic objective is constructed in this manner.

APOCHROMATIC OBJECTIVES

While achromatic objectives fulfil all ordinary purposes, they are not sufficiently corrected for critical

work, such as photography and resolution of minute objects, for which *apochromatic* objectives must be employed. These represent the highest degree of attainment of the optician's art, and are, in consequence, very expensive. Apochromats surpass all others in the matter of colour correction, and the essential factor in their construction is the use of the mineral *fluorite*. Fluorite possesses the following valuable optical properties :—

- (a) high degree of transparency ;
- (b) low refractive index ;
- (c) extremely small dispersion.

As a result of the use of fluorite at least three colours may be united, thus eliminating the secondary spectrum. This endows the objectives with a brilliance and "crispness" of image not attainable with the ordinary lenses, and enables the maximum resolving power to be obtained.

Apochromatic objectives must be used only in conjunction with "compensating" eye-pieces, and care must be taken to adjust the tube length carefully and to employ a highly corrected and properly centered condenser. The student or beginner in microscopy is not advised to purchase apochromats, as the modern achromatic or ordinary objectives have been brought to such a pitch of excellence that all routine examinations, and even research work, can be done with them.

CARE OF THE MICROSCOPE

The microscope is an instrument of precision, and care must be taken to preserve its accuracy. The instrument should be kept at a uniform temperature and not exposed to the sun or any source of heat. When not in use it must be protected from dust under a large bell-jar or in its box. Failing these, it should be covered with a clean duster or cloth. The microscope should be cleaned at intervals and its working surfaces very lightly smeared with the finest machine oil.

The oil-immersion objective must be cleaned each day after use by wiping the front lens with a well-

washed silk or cotton handkerchief. There is on the market a fine tissue paper known as "Lens Paper," which is very suitable for the purpose. Oil remaining on the lens-front dries and becomes sticky; later it becomes hard and is then difficult to remove. Canada balsam accidentally present on the lens from a mounted microscopic specimen may also dry hard in the same way. When cleaning the objective *do not use alcohol*, as the cement that unites the component lenses is soluble in alcohol, and in consequence the lens systems would become disorganised and the objective spoiled. Benzol or xylol must be used to remove dried oil, and if the oil is hard, repeated applications on a soft cloth are necessary.

Dry objectives—*e.g.* $\frac{2}{3}$ -in. and $\frac{1}{6}$ -in.—are cleaned with a piece of well-washed silk or fine cotton, or lens paper. If any oil or Canada balsam is accidentally present on the front lens it must be removed with a soft cloth moistened in benzol or xylol and the lens quickly dried with a soft cloth.

DIRECTIONS FOR USING THE MICROSCOPE WITH CONDENSER AND OIL-IMMERSION LENS

Before commencing to examine a specimen special attention must be paid to the following:—

- (1) The objectives and eye-piece must be clean.
- (2) The draw-tube must be adjusted to the correct length.
- (3) The flat side of the mirror must be used.
- (4) The condenser must be properly fitted into the sub-stage, so that it can be racked up practically flush with the stage. In microscopes where the condenser is inserted from below into a sleeve fitting, the condenser is often not properly pushed into place and cannot be racked up sufficiently high for its focus to be in the same plane as the specimen.

*For bacteriological work it is recommended that artificial light be always used.*¹ A gas-filled lamp with a frosted

¹ We do not propose to discuss the relative advantages of daylight and artificial light. It is sufficient to point out that

bulb is highly suitable. It is convenient to have some form of microscope lamp which partially encloses the bulb, so that no glare reaches the eyes. It is not advisable to use the microscope at a window, as the daylight entering the eyes renders the vision less acute. A suitable arrangement is to use the microscope on a small table at one side of the room so that the observer's back is towards the window. Not only is there less eye-strain, but it is much easier to work with both eyes open.

Focus the specimen with the low-power lens first. It is essential, particularly when examining tissues, to use the low-power first, in order to locate the organisms and also to observe the tissue reaction. Adjust the plane side of the mirror to the illuminant so that light is reflected into the condenser. Focus the specimen by means of the coarse adjustment. Manipulate the mirror until the image of the illuminant is seen in the centre of the field. Lower the condenser so that the whole field is evenly illuminated.

A suitable field having been obtained, the slide must be kept in place by means of the right-hand clip if a mechanical stage is not used. Rack up the objective a short distance, and place a drop of cedar-wood oil on the specimen below the objective. Screw up the condenser so that its upper surface is practically level with the stage, and be careful to see that the iris diaphragm is widely open. Turn the nose-piece until the oil-immersion lens is in position. With the eye at the level of the stage, lower the objective by means of the coarse adjustment until the lens is seen to dip into the oil. Apply the eye to the microscope and observe if the field is well illuminated. If not, adjust the mirror until the maximum illumination is secured.

daylight is inconstant and not always available, as in the winter, when artificial light must be employed. As the colours of certain preparations are different according to whether the illuminant is daylight or artificial light, the student is recommended to use a constant source of illumination—namely, artificial light.

Carefully focus down, using the *coarse* adjustment, and when the object has come into view, use the fine adjustment to secure sharp definition. It is often advisable to adjust the condenser at this stage so that the optimum illumination is secured.

The essential instructions for obtaining satisfactory results with the oil-immersion objective are : (1) employ a good artificial illuminant ; (2) use the plane side of the mirror ; (3) have the condenser racked up so that its focus is in the plane of the specimen ; (4) open the iris diaphragm to its widest ; (5) see that the field is well illuminated before attempting to focus the object.

When the observer has to examine a specimen for any length of time, as when searching for bacteria, he must adopt a comfortable position. Both elbows should rest on the table, and the slide is moved with the left hand while the right hand manipulates the fine adjustment.

EXAMINATION OF LIVING UNSTAINED ORGANISMS

“Hanging drop” preparations are used for this purpose.

A glass slide having a circular concavity ground in the centre is employed. By means of a match dipped in vaseline, a ring or square (according to the shape and size of the cover-glass) is outlined round the concavity. With a platinum loop place a drop of fluid containing the organisms on a cover-glass laid on the bench. Invert the slide over the cover-glass, allowing the glass to adhere to the vaseline, and quickly turn round the slide so that the cover-slip is uppermost. The drop should then be “hanging” from the cover-glass in the centre of the concavity.

Place the slide on the microscope, rack down the condenser slightly and partially close the iris diaphragm. With the low-power, focus the edge of the drop so that it runs across the centre of the field. Turn the high-power ($\frac{1}{8}$ -in. or 4 mm.) lens into position and

focus the edge of the drop. Obtain the best illumination by adjusting the condenser, and secure sharp definition by reducing the aperture of the diaphragm.

Motility of organisms can be detected in this way. It is advisable to use the high-power lens and not the oil-immersion objective. Owing to the viscosity of the oil, the cover-slip is apt to move during focussing and so cause currents which produce an appearance of motility in the organisms.

It is essential to distinguish between true motility, where the organism changes its position in the field, and Brownian movement, which is an oscillatory movement possessed by all small bodies (whether living or not) suspended in fluid.

DARK-GROUND ILLUMINATION

This method renders visible delicate organisms, such as the spirochaete of syphilis, which cannot be seen in unstained preparations with an ordinary microscope.

By means of a special condenser the specimen is illuminated only with oblique light. The rays do not enter the tube of the microscope, and, in consequence, do not reach the eye of the observer unless they are "scattered" as a result of the objects (*e.g.* bacteria) being of different refractive index from the medium in which they are suspended. As a result, the organisms appear brightly illuminated on a dark background.

Three requisites are necessary for adapting a microscope for dark-ground illumination :

- (1) A "dark-ground" condenser.
- (2) A suitable illuminant of sufficient intensity.
- (3) A stop which reduces the numerical aperture of the objective to less than 1.0, *if an oil-immersion lens is used.*

The Condenser.—A special condenser must be employed and may be of the paraboloid or of the concentric reflecting type. The latter, made by Leitz, has given excellent results in our hands. The object of the special condenser is to focus the light on the object,

the path of the rays being such that no direct light passes into the front of the lens. The illustration shows the path of rays through the concentric reflecting condenser. The condenser must be furnished with a centering device. It must be emphasised here that

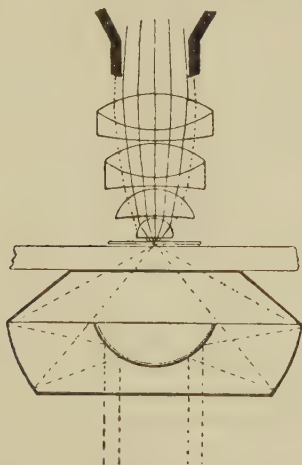


Diagram showing the path of rays through the condenser and a $\frac{1}{2}$ -in. oil-immersion lens fitted with a funnel stop (Leitz)

success with dark-ground illumination depends on the accurate centering of the condenser.

The Illuminant.—A lamp of sufficiently powerful intensity should be employed. The small arc lamp, or the “Pointolite” lamp, is well suited for the purpose. The arc lamp gives very good illumination and is supplied with a suitable condensing lens. The objection to it is that constant attention is required to keep the width of the arc adjusted as the carbons burn away. Arc lamps with a clockwork feed may be used to obviate this difficulty, but are apt to be unreliable. The Pointolite lamp requires a special resistance and works only on direct current; in addition, an enclosed chamber with a condensing lens has

also to be purchased. Its light is not so intense as that of the arc, but once adjusted it requires no further attention, and gives a constant light for any length of time. On the whole, the Pointolite is perhaps the more convenient illuminant.

The Funnel Stop.—When the objectives employed for dark-ground illumination have a numerical aperture of more than 1.0 (as in the case of oil-immersion lenses), a special stop to reduce the N.A. to less than 1.0 must be employed. This consists of a small funnel-shaped piece of vulcanite which screws into the objective behind the back lens. It is advisable to procure the stop from the maker of the lens employed. The stop is removed by unscrewing and the objective can at once be converted for ordinary use.

The thickness of the slide employed is important. The slides should be 1.0–1.1 mm. thick, and when a suitable supply has been obtained they should be used only for dark-ground work. The object to be examined must be at the focus of the condenser, the focal length of which is about 1.2 mm. If, therefore, too thick a slide is used, the focus of the condenser will be below the specimen and poor illumination will result; if the slide is too thin, the distance between the condenser and slide is such that a large amount of oil must be employed to make contact.

METHOD OF USING DARK-GROUND ILLUMINATION WITH THE OIL-IMMERSION OBJECTIVE

The microscope furnished with special condenser and funnel stop in the objective is placed in front of the illuminant. The condensing lens of the lamp is adjusted so that a parallel beam of light is obtained. With the plane side of the mirror the light is directed into the dark-ground condenser. Using the low-power ($\frac{9}{32}$ -in. or 16 mm.) objective, focus the surface of the condenser so that the engraved concentric rings on the surface come into view. These rings show the centre of the condenser and, if the condenser is out of centre—

as it probably will be—adjust the centering screws so that the rings become concentric with the edge of the field. The accurate centering of the condenser is of the utmost importance, and the time spent in this manipulation will be amply rewarded by the brilliant illumination obtained. The preparation to be examined must be covered with a No. 1 cover-slip, and it is advisable to ring round the cover-slip with vaseline to prevent evaporation. Place a large drop of immersion oil on the under surface of the slide or alternatively on the top lens of the condenser, and a similar drop on the cover-glass. Place the slide on the microscope stage, taking care that the top lens of the condenser is well below the slide. Rack up the condenser until oil-contact is made between the whole surface of the upper lens of the condenser and the slide; then bring the oil-immersion lens into position so that it touches the oil on the cover-slip. Adjust the mirror until the field, though dark, is evenly illuminated. Now carefully focus the specimen. A slight adjustment of the condenser, up or down, may be necessary, and some manipulation of the mirror may also be required. After a little practice an evenly illuminated field with an intensely dark background and brilliantly lit objects may be obtained with a minimum of trouble.

It is recommended that a microscope be reserved solely for dark-ground work, and kept ready with the illuminant in position, so that it is always available for immediate use. It is convenient to have the lamp and microscope fixed to a board for this purpose. The microscope, when not in use, should be covered with a bell-jar to exclude dust.

After use the condenser and objective should be carefully wiped free from oil. Discarded preparations should be dropped into a covered jar of benzol kept for the purpose. When a sufficient quantity has been accumulated it will be found that they can be very easily cleaned, as the oil and vaseline have been dissolved off in the benzol.

IV

CULTIVATION OF BACTERIA

BACTERIA cannot be fully identified in pathological specimens merely by their morphology and staining reactions except in certain cases—*e.g.* tubercle bacilli in sputum. It is usually necessary to grow them on artificial culture media in the laboratory, and the resulting growth is termed a *culture*. Furthermore, for purposes of identification it is essential that only one species or type of organism should be present in the growth, which is then referred to as a *pure culture*.

In order to study any particular micro-organism it is necessary therefore that a pure culture be obtained, and this usually involves three distinct operations :

- (a) The preparation of suitable artificial culture media.
- (b) The removal of other organisms from the medium and apparatus by sterilisation. As bacteria are ubiquitous, they are present in the material and on the articles used for making media. These contaminating organisms must be destroyed so that the culture medium is germ-free or sterile.
- (c) The cultivation of the organism and its isolation from others present in the pathological or other material. It is only occasionally, as by blood cultures, that organisms can be grown direct from the body in pure culture.

METHODS OF STERILISATION

Various methods are employed for the destruction of bacteria, according to the nature of the article to be sterilised ; and each method has its own particular use, with well-defined limitations. The usual

methods of sterilising are either (1) destruction of the organisms by some form of heat or by chemical poisons, (2) mechanical removal by filtration.

Chemical methods of sterilisation are not employed in the preparation of culture media, as the presence of the chemical which destroys the contaminating organisms will either kill or prevent the growth of the bacteria artificially introduced into the medium.

For the sterilisation of culture media and apparatus, heat is applied in some appropriate form, depending on the nature of the object to be sterilised.

STERILISATION BY HEAT

Objects may be sterilised by heat in two forms—dry heat (hot air or Bunsen flame) and moist heat (steam or hot water). Sterilisation by dry heat requires a much higher temperature, or a much longer time at the same temperature, than in the case of moist heat.

To ensure complete sterilisation, all forms of bacterial life must be destroyed, and the time of sterilisation is that necessary to kill the most resistant forms. Spores are extremely resistant to all methods of destruction, whether by means of heat or chemicals, and whereas the vegetative phase of an organism such as *B. anthracis* is killed by moist heat at 100° C. in a few seconds, the spores resist boiling for five minutes. The time for effective sterilisation, therefore, by any method is that which ensures complete destruction of all spores.

STERILISATION BY DRY HEAT

- (a) *Red Heat*.—Inoculating wires or needles, points of forceps, and searing spatulas are sterilised by this method, the heat from an ordinary Bunsen burner being utilised.
- (b) *Hot-Air Oven*.—This consists of a chamber having double walls between which hot air passes from a Bunsen burner. Modern hot-air ovens are heated electrically, and have an automatic device which keeps the temperature constant at any predetermined figure. A temperature of

160° C. for one hour or 180° C. for half-an-hour is necessary for complete destruction of bacteria and spores. It is obvious that such a temperature cannot be utilised for the sterilisation of culture media which contain water. This is the best method for sterilising *dry thin* glass ware, such as test-tubes, Petri dishes, flasks, throat swabs, quill tubes, graduated and capillary pipettes, and certain instruments, such as forceps and scissors. Test-tubes or flasks should be plugged with cotton-wool stoppers before sterilisation.

Certain precautions have to be observed when sterilising glass ware: (1) the glass should be perfectly dry, (2) the articles should be of thin glass and of even thickness to prevent cracking from uneven expansion, (3) the oven must be cold when the apparatus is inserted, then heated to the requisite temperature and kept at that temperature for the full time necessary for sterilisation; the oven is then allowed to cool before the articles are removed, as sudden or uneven cooling is also apt to cause cracking of the glass.

The advantage of this method is that all the articles are kept dry.

- (c) *Flaming*, by passing the article through the Bunsen flame without allowing it to become red hot. This method is useful for sterilising scalpels, needles, the mouths of culture tubes, cotton-wool stoppers, glass slides and coverslips. Needles and scalpels may also be sterilised by dipping them in methylated spirit and then burning off the spirit.

STERILISATION BY MOIST HEAT

- (a) *Boiling in a Water Bath*.—A suitable form of steriliser is the fish-kettle type made of enamel-ware. It should have a removable tray pro-

vided with a raised edge to prevent cylindrical instruments, such as syringes, from falling off. Five minutes at 100°C . is sufficient to kill all organisms; spores, however, may resist longer exposures, even up to $1\frac{1}{2}$ hours. This method of sterilisation has only a limited use in bacteriology, and is employed for tubes, instruments (forceps, scissors, etc.), syringes, pipettes, measuring cylinders, etc. A small amount of soda added to the water prevents rusting of steel instruments.

Note.—The inside of a test-tube may be rapidly sterilised by boiling water in it.

- (b) *Steam at 100°C .*—This method is extensively used in bacteriology. A Koch's steam steriliser heated by gas or electricity with an automatic regulator is employed, and is particularly useful for the sterilisation of culture media. Its advantages are: (1) the apparatus need not be costly; (2) both the container and the medium are sterilised; (3) as the medium is in an atmosphere of steam, there is no loss from evaporation; (4) the apparatus requires little or no attention.

Sterilisation is effected in two ways:

(1) One exposure for $1\frac{1}{2}$ hours. This ensures complete sterilisation, and can be used for such media as broth or nutrient agar. It cannot be used for nutrient gelatin, as this medium, after prolonged heating, loses its property of solidifying on cooling.

(2) Exposure at 100°C . for twenty minutes on each of three successive days. The accepted principle of this "intermittent" method of sterilisation is that one exposure is sufficient to kill the vegetative forms of bacteria; between the heatings the spores, being in a favourable medium, pass into the vegetative forms which are destroyed during the subsequent heating.

This method is very useful for sterilising media containing sugars which may be decomposed by higher temperatures or by prolonged heating. It is also employed for the sterilisation of gelatin media.

It is advisable to cover the cotton-wool stoppers of tubes or flasks with two or three layers of parchment paper or "Kraft" brown paper to avoid drenching.

- (c) *Steam at High Pressure in the Autoclave.*—The principle of the autoclave is that water boils when the vapour pressure is equal to the pressure of the surrounding atmosphere. If, therefore, the pressure be increased inside a closed vessel, the temperature at which the water boils will rise above $100^{\circ}\text{C}.$, the exact temperature depending on the pressure employed. The autoclave consists of a cylinder of gun-metal which is supported by a sheet-iron case. The lid is fastened down by screw clamps, and is rendered air-tight by means of an asbestos washer. The apparatus is furnished with a steam-tap and pressure-gauge, and also a safety-valve, which can be set to "blow off" at any desired pressure. The usual pressure employed is 15 lbs. per square inch above atmospheric pressure, making a total of 30 lbs. per square inch absolute pressure. With this pressure water boils at $120^{\circ}\text{C}.$, and fifteen minutes exposure to this temperature kills all forms of organisms, including the most resistant spores. This method of sterilising is used for such media as broth or nutrient agar, but must not be applied in the case of sugar media or gelatin, as the former are decomposed and the latter will not solidify on cooling.

Directions for using the Autoclave.—See that there is sufficient water in the bottom of the cylinder. Insert material to be sterilised and light the gas. Place the lid in position, see that the tap is *open*,

adjust the safety-valve to the required pressure¹ and screw down the lid. As the autoclave heats, air is forced out of the tap and eventually steam issues. Make sure that all air has been expelled from the cylinder and then turn off the tap. The pressure now rises until it reaches the desired level, when the safety-valve opens and the excess steam escapes. Allow the sterilisation to continue fifteen minutes *from this point*. When sterilisation is complete, turn out the gas and allow the autoclave to cool until the pressure-gauge indicates that the inside is at atmospheric pressure. Now open the tap and remove the lid. If the pressure is suddenly released, the liquid media, being at a temperature above 100° C. and suddenly exposed to ordinary atmospheric pressure, will boil furiously and be forced out of their containers with almost explosive violence. In order to avoid drenching by the steam, cotton-wool stoppers should be covered with parchment paper or "Kraft" brown paper.

STERILISATION AT LOW TEMPERATURES

The sterilisation of serum or body fluids containing coagulable protein is effected by heating for one hour at 57° C. on eight successive days. The principle is the same as in the case of intermittent sterilisation at 100° C. (*vide supra*). It is necessary to repeat the heating eight times to ensure complete sterilisation. Care must be taken not to allow the temperature to rise to 59° C., as inspissation will occur. The exposure to 57° C. is best carried out in a water-bath, but a 57° C. oven may be used.

Vaccines.—Vaccines should be sterilised in a water-bath at a comparatively low temperature, one hour at 60° C. being *usually* sufficient. Higher temperatures destroy bacterial toxin, and may also diminish the immunising power of the vaccine.

¹ In some varieties of autoclaves the adjustment of the safety-valve has to be determined previously by trial.

OTHER METHODS OF STERILISATION EMPLOYED FOR SPECIAL PURPOSES

STERILISATION BY CHEMICALS

- (a) Volatile antiseptics—*e.g.* chloroform. This method is often used in the sterilisation of serum (for culture media) and the chloroform, which is added in the proportion of 2 per cent., can later be removed by heating at 57° C.
- (b) Antiseptics of the phenol group. Lysol and cresol (5 per cent.) are powerful antiseptics. Their chief use in a laboratory is for sterilising surgical instruments and discarded cultures, and for disinfecting the hands and killing organisms accidentally spilt by the worker. Carbolic acid, 0·5 per cent., or tri-cresol, 0·3 per cent., is used for preserving sera and vaccines.
- (c) Metallic poisons. The most important in this group is a 1 in 1000 solution of perchloride of mercury, which is conveniently prepared from tablets. A bowl of this solution should be on every laboratory bench for sterilising the hands after finishing work.

STERILISATION BY FILTRATION

This is effected by the use of filters of unglazed porcelain (Chamberland, Maassen type) or of diatomaceous earth (Berkefeld, Mandler Clay Filter), the pores of which are so small that ordinary bacteria are prevented from passing through. The chief use of filtration is to obtain bacteria-free preparations of toxin, and for this purpose porcelain filters are to be preferred.

It is important that the filter candles should be tested before being used, as there are frequently cracks or flaws throughout the thickness of the candle. Filters should not pass a minute test organism, such as B. prodigiosus.

Further details are given on p. 280.

MAINTENANCE OF STERILITY

It is necessary that apparatus, after sterilisation, should be kept sterile.

Thus the interiors of test-tubes, flasks, etc., must be carefully protected from bacterial contamination due to access of air, dust, etc., before and after the addition of medium and during the subsequent cultivation of organisms.

Cotton-wool is extensively used in bacteriological work for stoppering tubes, flasks, bottles, etc. It is highly effective in excluding air bacteria. Cotton-wool plugs or stoppers should be about $1\frac{1}{2}$ in. long, 1 in. inserted into the mouth of the tube, etc., and $\frac{1}{2}$ in. projecting. They should fit firmly, but not so tightly as to render their removal difficult.

Tubes and flasks are generally protected from aerial contamination by means of cotton-wool stoppers, but if they have to be stored for any length of time, the stoppers should be covered with sterile brown paper, kept in place by means of fine string or a rubber band. Sterile rubber stoppers may, in some cases, be used instead of cotton-wool, particularly where the contents of the flask or tube have to be kept a considerable time, as in the case of immune sera; see also Blood Culture, p. 132.

Petri Dishes.—Each individual dish should be wrapped in “Kraft” brown paper before sterilisation, and kept in the paper until used. For a $3\frac{1}{2}$ -in. dish the size of paper should be 10 in. square. They may also be sterilised and kept in cylindrical copper boxes.

Pipettes.—1 c.c. and 10 c.c. graduated pipettes should be wrapped in a long strip of brown paper, which is wound round them in a spiral manner before sterilising. Bulb pipettes (10 c.c., 50 c.c., etc.) are also covered with brown paper. Under these conditions pipettes remain sterile in their wrappers for considerable periods of time.

Capillary pipettes are sterilised in large test-tubes,

15 in. \times 2½ in., having a cotton-wool stopper, or in copper boxes. The former method is preferable.

Ampoules are sterilised and kept in metal boxes.

PREPARATION OF CULTURE MEDIA

The majority of the organisms to be studied are pathogenic, and in order to obtain suitable growths the artificial culture media should approximate to the composition and reaction of the tissue fluid in which these organisms grow. Indeed for certain highly parasitic bacteria it is necessary either to add blood or serum to the culture medium or to grow the organisms actually in human transudate, such as ascitic or hydrocele fluid.

BOUILLON, NUTRIENT GELATIN AND AGAR

The basis for the media ordinarily employed in the study of the common pathogenic bacteria is nutrient broth, the function of the agar or gelatin being merely to solidify it.

BOUILLON

The first stage in the preparation is the making of a watery extract of meat. The type of meat used is an important factor in the quality of the broth obtained. Very young veal is best, but somewhat expensive. Fresh lean beef is quite satisfactory and much cheaper. Horse-flesh is cheaper still, but is usually not so fresh, and, in addition, contains a higher percentage of fermentable sugar, which makes the broth unsuitable for many purposes, such as the preparation of toxins (*vide* p. 181). The meat is carefully freed from fat, minced as finely as possible, and added to distilled water in the proportion of ½ kg. to 1 litre. After extraction for twenty-four hours at a low temperature—*e.g.* in the ice-chest—the mixture is strained through muslin to keep back the small particles of meat, and the meat residue expressed in a meat-press. The fluid is bright red in colour and there is often a thin layer of fat on the surface, which may be removed by skimming with a piece of filter paper. It is boiled for fifteen minutes,

or steamed in a Koch steriliser for two hours, when it becomes dark brown in colour and turbid on account of the alteration of the haemoglobin and the small particles of coagulated protein. It is now filtered and the clear fluid is made up to the original volume by the addition of distilled water. The unheated extract contains soluble proteins, salts, extractives and haemoglobin. As a result of the heating the meat proteins are coagulated, and are removed by filtration. The finished extract should be clear and light yellow in colour, but is not suitable as a culture media owing to the lack of nitrogenous material. Digested and uncoagulable protein in the form of commercial peptone is added in the proportion of 1-2 per cent., and the salt content is increased by the addition of sodium chloride ($\frac{1}{2}$ per cent.). These are dissolved by heat and the extract is again filtered. Owing to the sarcolactic acid present in the meat, the reaction of the extract is acid, and this reaction is unsuitable for the growth of most organisms. When the reaction has been adjusted to its optimum by suitable means (*vide* p. 58), the medium is sterilised in the autoclave, and Peptone Broth or Bouillon is the result.

Lab.-Lemco.—Recently there has been placed on the market a meat extract known as “Lab.-Lemco,” which may be used as a substitute for the extract of fresh meat in the proportion of 10 grams of Lab.-Lemco to a litre of water. The addition of salt ($\frac{1}{2}$ per cent.) and peptone (1 per cent.) converts it into ordinary bouillon. The reaction must be standardised as in the case of the ordinary meat-extract bouillon. In our experience Lab.-Lemco has given excellent results, and pneumococci may be grown quite abundantly in broth made from this material without the addition of serum. Lab.-Lemco is suitable for routine use, and effects a considerable economy in time and labour.

Bouillon being a fluid medium has certain disadvantages:

- (1) Growths do not show specially characteristic appearances, and therefore it is of little use in identifying species. Many organisms growing in this medium produce only a general turbidity.

- (2) Organisms cannot be separated from mixtures as, of course, individual colonies are not formed (*vide* p. 82).

It can, however, be rendered solid by the addition of gelatin, 10–15 per cent., or agar-agar, 2 per cent.

NUTRIENT GELATIN

This medium, first introduced by Koch, is made by dissolving the best sheet gelatin, 10–15 per cent. by weight, in broth. Solution is effected by heating in a steam steriliser. The mixture is filtered and, if necessary, cleared with white of egg (see Agar). The finished product is sterilised in the steam steriliser—twenty minutes each day on three successive days. Prolonged exposure to 100° C. or autoclaving destroys its property of solidifying when cooled, thus rendering it useless for bacteriological purposes.

The resulting medium is perfectly transparent when solid, and should be of firm consistence, yet not so stiff that it is split by the needle when inoculated (*vide* p. 78).

The proportion of gelatin used varies with the time of year, and in very hot summer weather 15 per cent. may be necessary. A suitable consistence may be obtained by adding 10 per cent. in the winter and 12 per cent. in the summer. Gelatin at this strength melts at 23° C., and is therefore fluid at incubator temperature. On this account it has a restricted use, and is employed only for organisms which grow at room temperature.

Gelatin is an albuminoid, and therefore is digested and liquefied by the proteolytic ferments of many bacteria. This property of liquefying gelatin is used as a means of differentiating organisms.

NUTRIENT AGAR

Agar-agar, or “Agar” for short, comes into the market in the form of dried strands of a seaweed found in the Chinese seas. It is also obtained as a powder, but the former is preferable.

The strands of agar are cut into small pieces by means of scissors, weighed, and added to the broth, which is then allowed to stand for some time. The broth is placed in the steam steriliser for two to three hours until the agar is thoroughly incorporated, and then filtered through Chardin filter paper either in the steriliser or in a hot-water funnel. Owing to the fine colloidal particles present, filtration is slow and the filtrate may not be perfectly clear. The medium should then be cooled, switched white of egg is added, and the whole reheated, when the egg albumen coagulates and incorporates the fine suspension. Subsequent filtration usually produces a clear medium. Nutrient agar may be sterilised either in the steam steriliser or in the autoclave.

This agar-broth medium is known as Nutrient Agar or "Ordinary Agar."

When fluid, it is perfectly clear, but when solidified is faintly opalescent. Its advantage is that it is solid at 37.5°C. , which is the optimum temperature for most pathogenic organisms. *The medium must be heated to 98°C. to melt it, but when melted it may be cooled down to 42°C. before solidifying.* This property is utilised in the preparation of serum agar or blood agar (*vide* p. 69). The agar may thus be cooled below the coagulating point of the serum proteins— 70°C. —and yet remain fluid so that the serum or blood is present unaltered in the medium.

When nutrient agar is to be enriched by the addition of body fluids, it should contain 3 per cent. of agar fibre.

Agar-agar differs from gelatin inasmuch as it is a carbohydrate and is not generally liquefied by bacteria.

Both broth and agar media may be enriched or modified by the addition of various substances.

GLUCOSE BOUILLON.—Bouillon + 1 or 2 per cent. of glucose. As glucose acts as a reducing agent, this medium may be used for the growth of anaerobes.

GLYCERIN BOUILLON.—Bouillon to which glycerin

is added in the proportion of 5–8 per cent. This medium is used for the growth of the tubercle bacillus.

Similarly, GLUCOSE AGAR is made by the addition of 1–2 per cent. of glucose, and GLYCERIN AGAR by adding 5–8 per cent. of glycerin to ordinary agar. The former is chiefly used for deep stab inoculation (*vide* p. 86) and the latter for growing the tubercle bacillus.

STANDARDISATION OF MEDIA

While many bacteria will show vigorous growth within a fairly wide range of reaction as regards acidity or alkalinity, there are others which require the reaction of the medium to be adjusted within narrow limits before multiplication takes place. Moreover, all organisms have a particular reaction at which the growth is optimum.

In order, therefore, to secure the best growth, particularly of the highly parasitic organisms, it is necessary that the adjustment of the reaction should be made as accurately as possible.

Two methods are given here—the one in which the acidity or alkalinity is expressed in relation to the neutral point of an indicator (phenol phthalein), and the other in terms of the absolute acidity which depends on the hydrogen-ion concentration. The latter method is the more satisfactory.

EYRE'S METHOD.—In this method the reaction is expressed as being acid or alkaline in terms of the neutral point of phenol phthalein. If acid, the prefix + is employed, and the sign – denotes alkalinity. The precise reaction is expressed by the number of c.c. of normal acid or normal alkali that is required per litre of medium in order to adjust it to the neutral point of phenol phthalein. Thus a reaction of + 20 indicates that the medium is acid (by the +) and that it requires 20 c.c. of normal caustic soda (N/1 NaOH) per litre in order to make it neutral to phenol phthalein. Similarly a reaction of – 8 shows that the medium is

alkaline and requires 8 c.c. of normal acid (N/1 HCl) per litre in order to render it neutral.

For the growth of most parasitic bacteria, the optimum reaction is about +10—i.e. acid to phenol phthalein.

Media were originally standardised by rendering them slightly alkaline to litmus. Litmus and phenol phthalein have not the same neutral point. Thus the neutral point of litmus corresponds to an acidity of +25 with phenol phthalein. Therefore the optimum reaction of media in general lies between the neutral points of these two indicators—i.e. *acid to phenol phthalein* and *alkaline to litmus*.

Method.—Place 20 c.c. of broth in an evaporating dish and add 20 c.c. of distilled water and a few drops of 0.5 per cent. solution of phenol phthalein in alcohol. As the mixture must be heated to remove carbon dioxide, the evaporating dish should be placed on a water-bath, or warmed on a tripod stand over a Bunsen burner.

Decinormal caustic soda (N/10 NaOH) is run in from a burette until the neutral point, as indicated by a faint but permanent trace of pink, is reached. Note the amount of alkali added, repeat the titration, and take the mean of the two readings. The calculation is as follows:—

Suppose y is this mean reading (in c.c. N/10 NaOH).
Then

20 c.c. of medium require	y c.c. N/10 NaOH
	to make it neutral to phenol phthalein.
1000 c.c. of medium require	$50y$ c.c. N/10 NaOH
	to make it neutral to phenol phthalein.
1000 c.c. of medium require	$5y$ c.c. N/1 NaOH
	to make it neutral to phenol phthalein.

The reaction is therefore $+5y$.

Example: Suppose $y=5.2$ c.c., then the reaction is +26.

26 c.c. of N/1 NaOH would therefore make 1000 c.c.

neutral to phenol phthalein, but the required reaction is + 10, so that 26–10 c.c.—*i.e.* 16 c.c. of N/1 NaOH—must be added per litre to give this reaction.

While + 10 is a suitable reaction for most organisms, some bacteria, such as the gonococcus and meningococcus, grow best in a slightly more alkaline medium having a reaction of + 6.

The disadvantage of Eyre's method is that, while it seems sound in theory, the practical application shows a wide margin of error. This is due to the fact that the "end-point" of the indicator is not sharp owing to the presence of phosphates in the medium. These act as "buffer salts," so that near the neutral point the addition of acid or alkali only produces a gradual change in the colour of the medium. The precise end-point cannot be determined with any degree of accuracy, as the personal factor predominates, depending on the colour sense of the manipulator and what he considers an adequate colour change.

STANDARDISATION ACCORDING TO HYDROGEN-ION CONCENTRATION.—The true acidity of any fluid depends on the number of dissociated hydrogen ions present, and the reaction of the medium is dependent on and measured by the hydrogen-ion concentration. The greater the concentration, the more acid the medium.

The standard of hydrogen-ion concentration is N/1 acid (*e.g.* N/1 HCl), and, if we assume this is fully dissociated, contains 1 gram of hydrogen ions per litre. Then N/10 is only $1/10$, or 10^{-1} , of this concentration, N/10,000 is 10^{-4} , and N/10,000,000 is 10^{-7} , of the standard hydrogen-ion concentration.

The hydrogen-ion concentration is expressed in the form of the "hydrogen exponent" designated as pH, which is the logarithm of the concentration as compared with the standard, omitting the negative sign.

Thus N/10,000 HCl has a concentration of 10^{-4} and is represented thus—pH 4.0, while N/100,000,000, or 10^{-8} , has a pH 8.0.

It is therefore apparent that as the hydrogen exponent (pH) increases, the acidity decreases, and that unit difference of pH means ten times difference in the actual acidity.

Thus N/1000 HCl has a pH 3.0 and is ten times as acid as N/10,000 HCl, of which the pH is 4.0.

$$\text{pH} \propto \frac{1}{\text{concentration of H-ions}} \propto \frac{1}{\text{acidity}}$$

The absolute determination of pH is made by means of the hydrogen electrode, and it is found that the concentration of hydrogen ions in specially purified water is 10^{-7} at 20°C .—*i.e.* pH 7.0. The H-ion concentration of blood plasma has a pH 7.5, and this is the optimum reaction for the growth of pathogenic micro-organisms.

It is not practicable to use the hydrogen electrode for general laboratory use, and a simple and satisfactory method has been devised whereby media can be adjusted to any desired pH. The method depends on the fact that when a fluid contains buffer salts such as phosphates, the addition of alkali or acid does not cause an abrupt change in the colour of an indicator. For example, phenol sulphone-phthalein (or phenol red) is yellow in acid solution and purple-pink in alkaline solution. If an alkali be gradually added to an acid phosphate solution containing phenol red, the change in colour will commence at pH 6.8, and the colour will become more purplish pink, until the final change is reached at pH 8.4; thus the "range" is pH 6.8–pH 8.4, and as it covers the optimum reaction for culture media—namely, pH 7.5—this particular indicator is used.

Other dyes have their own definite range of pH in which colour change occurs. Thus the range of Thymol Blue is pH 1.2–2.8, of Brom-Cresol Purple pH 5.2–6.8, and there is now available a complete series of indicators which exhibit colour changes between pH 1.0 and pH 10.0.

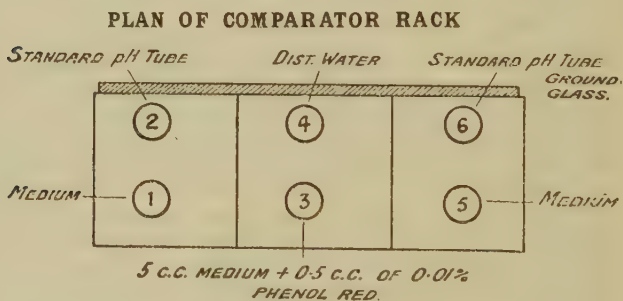
The procedure for adjusting culture media to a

definite pH is comparatively simple. Solutions of fixed and known hydrogen-ion concentrations are prepared and to each solution a definite amount of indicator (for this purpose phenol red) is added. The resulting tint is the standard to which the medium must be brought by titration with alkali, and so the amount of alkali to be added per litre may easily be calculated.

Apparatus required:

1. A set of tubes of standard bore containing solutions of known pH to which the indicator has been added. These solutions are made up by mixing N/15 Na_2HPO_4 and N/15 KH_2PO_4 solutions in certain proportions. These standard tubes, which have a range of pH 6.6–8.0 at intervals of pH 0.2, are best purchased ready made up. Details of their preparation may be found in larger works.

2. A special comparator rack.



3. "Cordite" tubes, which have a uniform thickness of wall and bore, and are identical with the tubes containing the standard solutions.

4. A solution of phenol red, 0.01 per cent., in distilled water.

5. N/20 NaOH made up as follows:—

500 c.c. N/10 NaOH

91 c.c. 0.01 per cent. phenol red

distilled water to 1000 c.c.

6. A burette, preferably a microburette, measuring to 0.01 c.c. (An emergency one may be made from a 1 c.c. graduated pipette, a short piece of rubber tubing, a glass tube drawn out to a fine point, and a pinch-cock.)

Tube 3 contains 5 c.c. of the medium + 0.5 c.c. of 0.01 per cent. solution of phenol red.

Tubes 1 and 5 contain the medium only.

Tube 4 contains distilled water only.

Tubes 2 and 6 are the standard tubes for comparison. By this arrangement of the tubes the colours of 1 and 2, and of 5 and 6 are superimposed when examined in the rack.

It has been found easier to bring the solution to a tint midway between two standard colours than to make the tint match a given standard. Thus suppose a reaction of pH 7.5 is required, then standard tubes pH 7.4 and pH 7.6 are placed in positions 2 and 6 of the rack.

The N/20 NaOH solution is run into tube 3 until the tint produced is midway between the tints of tubes 2 and 6, and the amount noted. The average of two readings is taken and the calculation is as follows:—

Let the number of c.c. of N/20 NaOH = y .

5 c.c. medium require y c.c. N/20 NaOH
to adjust reaction to pH 7.5.

1000 c.c. medium require $200y$ c.c. N/20 NaOH
to adjust reaction to pH 7.5.

1000 c.c. medium require $10y$ c.c. N/1 NaOH
to adjust reaction to pH 7.5.

Example: Suppose $y = 1.15$, then 11.5 c.c. N/1 NaOH are required per litre of the medium to adjust the reaction to pH 7.5.

It will be readily seen that the tint due to the mixture of the medium and the indicator in tube 3 is compensated for by the medium in tubes 1 and 5.

The indicator is added to the standard alkali solution, so that when the medium in tube 3 is titrated, the actual concentration of the dye always remains constant.

The standardisation of a solid medium such as nutrient agar presents greater difficulty than in the case of fluid media. The medium may be titrated when liquid, but the exact determination is not easy to obtain with any degree of accuracy. It has been found, however, that agar of good quality has very little effect on the reaction of the broth to which it is added, especially if the agar, after weighing, has been previously washed. The reaction of the finished agar may be controlled by titrating the melted medium and then comparing the colour when cold. Gelatin may be conveniently adjusted if the medium is liquefied and kept at about 37° C.

PEPTONE WATER

This is a simple medium, consisting of

Peptone 1 per cent.

NaCl 0.5 „

dissolved in warm water and then filtered. It is sterilised in the autoclave. It is chiefly used as the basis for sugar fermentation media, as broth and agar, being made from meat, contain a small amount of muscle sugar, and it is essential that the basic medium, to which various carbohydrates are added for fermentation tests, should be free from natural sugar.

Peptone water is used to test the formation of indol (see p. 200), and also for the enrichment of the *Vibrio cholerae*, when isolating this organism from infected material. In the latter case the medium should be adjusted to a reaction neutral to phenol phthalein (approximately pH 8.0), as the *Vibrio cholerae* prefers an alkaline medium.

PEPTONE WATER AGAR.—This consists of peptone water solidified with 2 per cent. agar, and is

used as a basis for solid media containing sugar (*vide* p. 172).

SUGAR MEDIA

Under the designation of "sugars" are included a variety of fermentable substances, chiefly carbohydrates, which are used in the identification and classification of organisms.

These substances are fermented with the formation of acid, and, if the process continues, the acid is split up and gas is formed in addition.

The medium consists of peptone water, to which the fermentable substance is added in the proportion of 1 per cent. An indicator is added to denote acid change. This may be litmus solution (Kubel-Tiemann), neutral red (0.25 per cent. of a 1 per cent. solution), or Andrade's indicator (1 per cent.).¹ If acid is produced, the litmus turns bright red; the neutral red, pink; and Andrade's indicator, reddish pink. In order to detect gas, a small inverted tube is placed in each culture tube (Durham's fermentation tube). During the process of sterilisation the heat drives out the air from the inverted tubes, which should be completely filled with liquid and contain no air bubbles when the tubes are cool.

The method of making the medium is as follows:—

The stoppered test-tubes containing the small inverted tubes are sterilised by dry heat in the hot-air oven. The peptone water (with the indicator added) is sterilised by autoclaving. The sugars are made up separately in 10 per cent. solutions, which are sterilised in the steamer. The requisite amount of sugar solution is added to the peptone water. The medium is tubed (*vide* p. 78), and steamed for twenty minutes on three successive days.

The fermentable substances most commonly used are the following:—

¹ Made by adding sodium hydrate to a $\frac{1}{2}$ per cent. solution of acid fuchsin until the colour just becomes yellow.

Monosaccharides :(a) *Pentoses*—

- (1) Arabinose (from gum arabic).
- (2) Xylose (from wood).
- (3) Rhamnose.

(b) *Hexoses*—

- (1) Glucose.
- (2) Laevulose.
- (3) Mannose (from the ivory nut).
- (4) Galactose (made by the hydrolysis of lactose).

Disaccharides :

- (1) Saccharose (Cane Sugar).
- (2) Maltose (Malt Sugar).
- (3) Lactose (Milk Sugar).

Trisaccharide :

Raffinose.

Polysaccharides :

- (1) Starch.
- (2) Inulin (from dahlia tubers).
- (3) Dextrin.

Alcohols :

- (a) *Trihydric*—Glycerol (Glycerin).
- (b) *Tetrahydric*—Erythritol (Erythrite).
- (c) *Pentahydric*—Adonitol (Adonite).
- (d) *Hexahydric*—Mannitol (Mannite).
Dulcitol (Dulcite).
Sorbitol (Sorbite).

Glucosides (vegetable products which on hydrolysis yield a sugar):

- (1) Salicin.
- (2) Coniferin.

Non-carbohydrate Substances :

- (1) Inosite—a benzol compound.

SERUM AND BLOOD MEDIA

These may be divided into two classes :

- (1) Where the serum or blood is coagulated by heat (above 70° C.) and a solid medium results.
- (2) Where the serum or blood is added in fluid form to enrich simpler media.

MEDIA CONSISTING ALMOST ENTIRELY OF SERUM
OR BLOOD

LOEFFLER'S BLOOD SERUM.—To ox, sheep or horse serum is added one-third of its volume of 1 per cent. glucose broth. The mixture is added to stoppered sterilised tubes, which are laid on a sloped tray and placed in the serum inspissator. The temperature is then raised to 75°–80° C., when the serum coagulates to a yellowish white solid. The tubes are thereafter sterilised at 90° C. (in the top of the steam steriliser) for twenty minutes on each of three successive days. It is essential that overheating be avoided, as the expansion of air bubbles and the formation of steam from the fluid droplets in the partially solidified material lead to the disruption of the medium.

Loeffler's serum is especially useful for the growth of the diphtheria bacillus. Not only does it produce a luxuriant growth in a short time (twelve hours), but it is also valuable in eliciting the specific staining reaction of the organism by Neisser's method (*vide* p. 105).

Collection of Blood.—A sterile wide-mouthed stoppered bottle is taken to the abattoir at a time when animals, preferably sheep, are being killed. After the neck vessels have been severed, the blood is allowed to flow for some time and then the stream from the carotid artery is allowed to spurt direct into the bottle. When filled, the bottle is stoppered and returned carefully to the laboratory. The clot is then separated from the sides of the bottle by means of a stiff sterile wire. The blood is kept overnight in the ice-chest and the clear serum then pipetted off. With care contamination can be avoided.

Defibrinated blood is collected in a similar way. The stoppered bottle, however, contains glass beads. The bottle is only half filled, and immediately a sufficient quantity of blood has been collected the stopper is replaced and the bottle continuously shaken for about

five minutes. The blood so treated does not clot on standing.

Sterile specimens can be obtained by inserting a cannula or wide-bore needle into the external jugular vein. If a sheep is selected, the wool is clipped from the side of the neck and the part shaved. Contamination can be minimised by placing a bag made of water-proof material over the head of the animal. It is best to use a cannula connected by rubber tubing to a stoppered flask or bottle, the whole being enclosed in brown paper and sterilised. The vein may be made prominent by pressure on the lower part of the side of the neck. The skin over the vein is carefully sterilised with soap and water and then alcohol. The cannula is inserted into the vein and the requisite amount of blood removed. Horses are treated similarly, except that it is advisable to make a small incision with a sharp knife in the skin over the vein. The cannula is then more easily introduced.

COAGULATED BLOOD MEDIUM.—Defibrinated blood is obtained as above, tubed and coagulated, and thereafter sterilised in exactly the same manner as Loeffler's blood serum. This medium is chocolate in colour.

HISS'S SERUM WATER.—As certain pathogenic organisms—*e.g.* streptococcus, pneumococcus—will not grow well in ordinary sugar media, it is necessary for fermentation tests to use a medium containing serum.

One part of serum is mixed with three parts of distilled water, and 1 per cent. of litmus solution is added as an indicator. The various sugars are incorporated in the proportion of 1 per cent. This medium does not coagulate on heating, and may be sterilised in the steamer in the same way as other sugar media—namely, twenty minutes each day on three successive days.

Fermentation is indicated by the production of acid, which alters the indicator and causes coagulation of the medium.

MEDIA ENRICHED WITH SERUM OR BLOOD

These media are used for certain delicate pathogens, such as the pneumococcus, gonococcus and meningococcus, which do not grow well or completely fail to grow on ordinary media.

SERUM AGAR.—Ordinary nutrient agar containing 3 per cent. agar + 5 per cent. of sterile uncoagulated serum. Animal serum is quite satisfactory, and can be obtained in the laboratory by bleeding a rabbit (*vide infra*), allowing the blood to coagulate in a sterile stoppered measuring cylinder, and removing the serum after it has fully separated. All the necessary precautions must be taken to avoid contamination. Serum can be stored in sealed tubes after heating at 57° C. on three successive days, but fresh serum yields much better results than heated serum in the culture of certain pathogens—*e.g.* gonococcus.

The agar is first melted and then cooled to about 50° C. The serum is usually added to the agar in tubes, and, after it is incorporated, the medium is either solidified in the form of slopes, or poured into Petri dishes (*vide p. 78*). As serum agar is indistinguishable from ordinary agar, the tube or dish should be marked “+ S.”

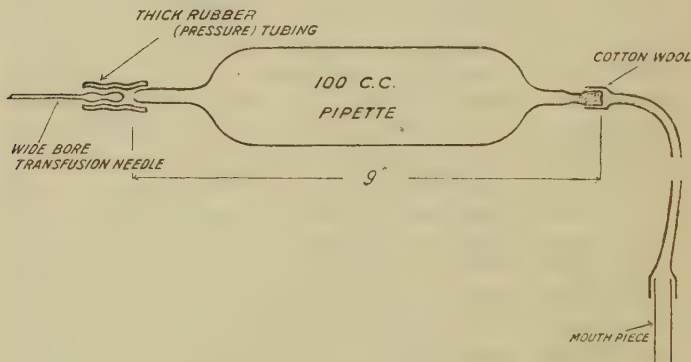
Sterile hydrocele fluid or sterile ascitic fluid, withdrawn aseptically, may be used instead of serum.

SERUM-SMEARED AGAR.—This is made by running a few drops of sterile serum on the surface of an agar slope or plate (*vide p. 78*). This medium is not so satisfactory as the preceding one, but is useful in an emergency.

BLOOD AGAR.—This is an important medium and is specially suitable for the gonococcus, the haemophilic group of bacteria (*e.g.* Pfeiffer's bacillus) and other delicate pathogens.

Either human or animal blood is suitable. Defibrinated rabbit blood obtained by cardiac puncture can be recommended for general use. The animal is fastened to a board and the fur clipped over the left

side of the chest; the area is shaved and then sterilised with alcohol and ether. A 100 c.c. bulb pipette (see diagram) is cut down at both ends to 9 in. in length, one end being slightly tapered and the other end stoppered with cotton-wool. It is wrapped in brown paper and sterilised in the hot-air chamber. A wide-bore transfusion needle is fitted into a short length ($1\frac{1}{2}$ in.) of thick rubber tubing and sterilised by boiling. When the animal is anaesthetised, the rubber



tubing is attached to the tapered end of the pipette and to the other end is fitted a mouth-piece such as that used in pipetting (*vide* p. 134). The needle is inserted into the left side of the chest and suction applied. The needle should lie in the right ventricle of the heart, and blood rapidly flows into the pipette. About 50 c.c. of blood per kilo. of body-weight can be obtained. The blood is then transferred to a sterile 500 c.c. flask containing glass beads. Agitation should be kept up for at least five minutes, to ensure that all the fibrin is separated. Human blood may easily be obtained by means of vein puncture (see blood culture, p. 131). The blood is added to melted 3 per cent. agar at 50°C . in the proportion of 5–10 per cent., as in preparing serum agar (*q.v.*). For special purposes as much as 20 per cent. of blood may be added.

BLOOD-SMEARED AGAR.—This is a useful emergency medium, on which *B. influenzae* will grow quite well. The skin at the base of the nail of the left thumb is sterilised by rubbing vigorously with soap and water, and then with alcohol which is allowed to evaporate. A puncture is made with a Hagedorn needle sterilised by flaming, and the resulting drop of blood is taken up with a platinum loop and spread on an agar slope, which is kept horizontal until the blood has coagulated.

DORSET'S EGG MEDIUM

This medium is used for growing the tubercle bacillus. Four fresh eggs are beaten up and 25 c.c. distilled water then added. The mixture is strained through muslin to remove air bubbles, run into sterile tubes (*vide* p. 78), and solidified in the sloped position in the serum inspissator at 75°–80° C. The tubes are then sterilised at 90° C. (at the top of the steam steriliser) for twenty minutes each day on three successive days.

All apparatus used should be sterile, and the eggs, before they are broken, should be placed for a few minutes in alcohol, which is then allowed to evaporate.

The addition of sufficient basic fuchsin to the medium to render it pale pink is advisable, as early growths of the tubercle bacillus are thus more easily seen.

Note.—As the tubercle bacillus may take some weeks to grow, the tubes are sealed after inoculation by pushing down the cotton-wool stopper below the top of the tube, and pouring in a little melted paraffin wax.

GLYCERIN EGG MEDIUM (for growing the human type of tubercle bacillus).—One part of 6 per cent. glycerin in 0.85 per cent. saline solution is added to 3 parts of beaten egg which is then treated as above.

LITMUS MILK

Used in testing for the fermentation of lactose.

Fresh milk is steamed for twenty minutes and then allowed to stand for twenty-four hours in order that the cream may separate. The milk is siphoned off and

coloured with litmus. Amounts of 10 c.c. are placed in tubes, which are then sterilised by steaming for twenty minutes each day for three successive days.

POTATO SLOPES

This medium is not often used. Large potatoes are selected, carefully washed and peeled. By means of a potato borer (or large cork borer) a cylinder of potato is obtained. This is cut obliquely and each half is placed in a test-tube with the thick end resting on a plug of cotton-wool, or in a special potato tube. The tubes are sterilised by steaming for $1\frac{1}{2}$ hours.

GLYCERIN POTATO.—Potato slopes in tubes are covered with 6 per cent. solution of glycerin in water and steamed for half-an-hour at 100°C . The glycerin is decanted and the tubes are then steamed for one hour. This medium is sometimes used for the cultivation of the tubercle bacillus.

MEDIA FOR SPECIAL PURPOSES

MACCONKEY'S BILE-SALT NEUTRAL-RED LACTOSE AGAR

This is a valuable medium for differentiating intestinal organisms of the coli-typhoid group.¹ It is a peptone solution solidified with agar, to which bile salt, $\frac{1}{2}$ per cent., and lactose, 1 per cent., are added, with neutral red as the indicator.

<i>Add</i>	Peptone	2 per cent.
	Sodium taurocholate	$\frac{1}{2}$ „

to water and dissolve by heat. Then add agar fibre, 2 per cent., and dissolve in the steamer or autoclave. Filter, and if necessary clear with white of egg. Add a sufficient amount (about 1:400) of a freshly prepared 1 per cent. solution of neutral red to give the medium a distinct reddish brown colour. If the medium is acid and assumes a rose-pink colour, add

¹ Other differentiating media—*e.g.* Endo's—are in common use, but in our experience we have found MacConkey's medium the most suitable.

caustic soda solution until the colour becomes definitely reddish brown. The medium is then sterilised in the autoclave and finally 1 per cent. lactose (previously sterilised separately in a 10 per cent. watery solution) is incorporated. The completed medium is sterilised as in the case of other sugar media.

Organisms which produce acid from lactose—*e.g.* *B. coli*—form rose-pink-coloured colonies, whereas the colonies of non-lactose-fermenters—*e.g.* *B. typhosus*—are colourless.

DIEUDONNÉ'S MEDIUM

This is an alkaline blood medium used for the isolation of the cholera vibrio.

Equal parts of defibrinated ox blood and normal solution of caustic soda (40 grams per litre) are steamed in the Koch steriliser.

Volatile ammoniacal substances are formed in the blood-alkali mixture, and these must be eliminated. This can easily be done by repeated steaming until the mixture is free from ammoniacal smell.

Three parts of this mixture are added to seven parts of 3 per cent. agar.

SMITH-NOGUCHI MEDIUM

This medium was introduced by Theobald Smith and developed by Noguchi, who has used it with considerable success for the growth of pathogenic spirochaetes. It consists of a small piece of fresh sterile animal tissue, preferably rabbit kidney, covered with a long column of ascitic fluid on which is superimposed a layer of vaseline.

The medium is anaerobic, the function of the fresh tissue being to destroy by means of its catalase any hydrogen peroxide that might be formed by organisms, and which is detrimental to their growth.

The essentials for success with this medium are :

(a) Good technique.

(b) Rigid asepsis in removing and manipulating the rabbit kidney.

- (c) Suitable ascitic fluid: this must be clear, free from bile, and of high specific gravity; it must be sterile from the beginning, as sterilisation either by heat (57°C.) or by filtration through an earthenware filter renders it unsuitable.

Technique.—Special long thin tubes ($8 \times \frac{1}{2}$ in.), known as Noguchi tubes, are employed. These should be cleaned, stoppered with cotton-wool and sterilised in the hot-air chamber. They are then wrapped up in batches of twenty-five in towels and autoclaved. Eight pairs of forceps and eight pairs of scissors are sterilised. It is advisable to keep these instruments solely for the purpose, and they are best sterilised in three large test-tubes $8 \times 2\frac{1}{2}$ in., plugged with wool, in the hot-air steriliser. One tube contains four pairs of scissors and four pairs of forceps, and the other two tubes each contain two pairs of scissors and two pairs of forceps.

A large healthy rabbit (1500–2000 grams in weight) is selected, and fastened to a board. It is then bled by cardiac puncture (*vide* p. 69). It is essential that as much blood be removed as possible, so that the piece of kidney added to the medium is bloodless. The animal is now immersed completely in a bath of weak lysol (5 per cent.) for ten minutes, placed on the board and surrounded with towels soaked in weak lysol or 1:1000 perchloride of mercury solution, so that only the abdomen is exposed. For removing the kidneys the tube with the four pairs of scissors and forceps is used. The operation should now be done under the inoculating hood (p. 82). With a sterile knife and a pair of forceps make an incision the length of the abdomen and widely dissect away the skin. With another pair of scissors and forceps open the abdominal cavity and draw the intestines to one side. With fresh instruments remove the kidney from one side and place it in a sterile Petri dish. With the forceps pull over the intestine to the other side and with another set of instruments remove the other kidney in a similar

manner to a second Petri dish. The animal should then be thoroughly examined for any intercurrent infection, such as snuffles, pneumonia, etc., and kidneys should be retained only if the rabbit is perfectly sound.

The kidneys are cut up into small pieces, using a separate tube of instruments for each organ. The capsule is stripped and the fat at the hilum removed. Each kidney yields eight to eleven pieces, which are taken up with the second pair of forceps, and one piece is placed in each Noguchi tube. Sterile ascitic fluid is run in by means of a 50 c.c. bulb pipette until the tubes are half full. Vaseline, previously sterilised by autoclaving in a 250 c.c. conical flask, is melted and added by means of a 10 c.c. pipette, forming a layer of about half-an-inch. The tubes are then incubated overnight and examined for contamination. This is evidenced by turbidity and often gas formation. Such tubes are of course rejected. It is only very occasionally that contaminations occur if the above technique is used.

The tubes are inoculated by first melting the vaseline and then introducing the inoculum to the bottom of the tube by means of a capillary pipette and rubber teat. Material is similarly withdrawn to be examined. Growth of such organisms as the *Spironema pallidum*, *Bacterium pneumosintes*, "globoid bodies" of poliomyelitis is evidenced by a clouding of the fluid at the bottom of the tube, which appears about the fourth to the tenth day of incubation. Turbidity, however, is not definite evidence of growth, as uninoculated control tubes also may show clouding just above the piece of tissue.

N.N.N. (NOVY, MACNEAL, NICOLLE) MEDIUM

For the growth of *Trypanosomes* and *Leishmaniae*.

Meat extract is made in the ordinary way with rabbit or beef flesh, using 125 grams per litre of water. To this are added peptone, 20 grams; sodium chloride, 5 grams; agar fibre, 20 grams; and 10 c.c. normal sodium carbonate solution. After tubing, autoclaving

and then cooling to 50° C., there is added to the medium in each tube twice its volume of defibrinated rabbit blood (see p. 69). The contents of the tubes are mixed by rotation between the palms of the hands and allowed to solidify in the sloped position, preferably on ice. It is important to obtain a large amount of water of condensation.

Before inoculation the tube is placed in the upright position, and material is usually introduced by means of a capillary pipette. The growth of *Leishmaniae* occurs mostly in the water of condensation.

LEPTOSPIRA MEDIA

These were introduced by Noguchi for the cultivation of the causal organisms of Infectious Jaundice and of Yellow Fever. These media are rendered semi-solid by the addition of one-tenth of their volume of 2 per cent. nutrient agar.

- (1) Rabbit serum, 2 parts; saline or Ringer's solution,¹ 6 parts; citrated rabbit's plasma, 1 part; neutral 2 per cent. agar, 1 part. The last is added fluid at 60° C. and the contents of the tubes are mixed by rotation to ensure a uniform distribution.
- (2) In a Noguchi tube place 8 c.c. saline or Ringer's solution at 55° C. Add 1 c.c. melted nutrient agar. Then add 1 c.c. defibrinated rabbit's blood and allow the medium to cool. This medium is applicable when the organisms have been accustomed for some time to artificial cultivation.

SABOURAUD'S MEDIUM

A medium for the growth of fungi, consisting of

Peptone	.	1 per cent.
Maltose	.	4 ,,
Agar	.	1.8 ,,

which is made up as in the case of ordinary nutrient agar.

¹ Sodium chloride 9 grams, calcium chloride 0.25 gram, and potassium chloride 0.42 gram, per litre.

ROBERTSON'S BULLOCK HEART MEDIUM

This medium is used for the cultivation of the anaerobes of the gas gangrene group. It is also spoken of as "*cooked meat medium*."

Bullock heart, freed from fat, is finely minced and 8 oz. added to 8 oz. of tap water. The mixture is slowly heated and the meat thoroughly cooked. Normal caustic soda is added until the reaction is distinctly alkaline to litmus. The medium is distributed into tubes, and a layer of liquid paraffin $\frac{1}{2}$ in. deep is run on to the surface. Sterilisation is effected by steaming or autoclaving. Inoculation or removal of material is done by means of a capillary pipette.

ALKALINE EGG MEDIUM

Beat up the yolk of one egg and the whites of two, adding 6 c.c. of N/1 caustic soda. Transfer this to a large flask and make up to 500 c.c. with tap water. Heat slowly to 95° C. for 1½ hours, then filter through cotton-wool and distribute into tubes. Cover the medium with a layer of liquid paraffin and autoclave. A clearer medium is obtained by using the whites of two eggs without any yolk, adding 4 c.c. of normal caustic soda, and making up to 330 c.c. with tap water.

USE OF CULTURE MEDIA

Only general methods are described here. Special methods applicable for particular purposes are referred to in the appropriate sections—*e.g.* under special media.

SOLID MEDIA

Medium, after preparation, is usually kept (in bulk) in flasks or bottles stoppered with cotton-wool. For immediate use it is allowed to solidify in sterile stoppered test-tubes either by cooling after having been melted by heat, as in the case of agar or gelatin (*vide* p. 57), or by coagulation in an inspissator, as in

the case of solidified serum or egg media (*vide* p. 67). The tubes are plugged with cotton-wool, and sterilised in the hot-air oven before the addition of the medium.

Tubing of medium is conveniently carried out by means of a sterile 6 in. glass funnel (fixed in a burette stand) with a short length of rubber tubing and glass delivery nozzle fitted to the stem and controlled by a pinch-cock. During the tubing the funnel is covered with the lid of a large sterile Petri dish to avoid aerial contamination.

Depending on the method of inoculation to be used, solid media are solidified in tubes as follows :—

(a) *Upright, for “puncture” or “stab” culture.*—

The test-tube is half filled with the medium, which is allowed to solidify in the upright position. It is inoculated by plunging a long straight wire (*vide* p. 80), charged with the material, vertically down the centre of the tube. This method is used for anaerobic cultures in glucose agar, and for testing the liquefaction of gelatin.

(b) *Sloped, for “stroke” culture.*—This is often called a “*slope*” or “*slant*,” and ensures a maximum surface of the medium exposed to the air. Quantities of 5 c.c. of medium for an ordinary $6 \times \frac{5}{8}$ in. tube are sufficient. When a large number of agar tubes have to be sloped, special trays, which allow the tubes to be laid at the correct angle, are useful, and moreover they can be stacked one upon another so that very little bench space is required during solidification. Fresh agar slopes, after cooling, contain “water of condensation” at the foot of the tube, and the tubes should be stored and handled in the vertical position to prevent the fluid from flowing over the surface of the medium.

Plates.—Where a large surface is necessary, as in the separation of organisms from mixtures (*vide* p. 82), the medium—*e.g.* agar or gelatin—is allowed

to solidify in the form of a thin layer in a Petri dish. For a dish of $3\frac{1}{2}$ in. diameter, 12 c.c. of medium are ample. The *melted* medium is poured into the dish with the necessary precautions to avoid contamination. Medium which has been tubed—*e.g. vide supra* (a)—can be melted and used for pouring plates.

Shake Cultures.—Agar or gelatin medium in tubes—*e.g. (a), vide supra*—is inoculated in the melted condition at a temperature which keeps the medium fluid, but is not *immediately* lethal to the organisms inoculated (*e.g. 50° C.*). The contents of the tubes are mixed by rotation between the palms of the hands and then poured at once into a Petri dish, as in water examination (*vide p. 151*), or left to solidify in the tube so that colonies may develop in the depth of the medium, as when separating anaerobes. In the latter case the test-tube is filed and broken, and the colonies “picked out” of the medium exposed in this way.

FLUID MEDIA

Fluid media are used in (1) test-tubes plugged with cotton-wool, the tubes being about half filled, or (2) plugged flasks, according to the quantity of medium required for individual cultures.

INOCULATION OF CULTURE MEDIA

According to the nature of the medium and the inoculum, various methods are employed for inoculation, and the following instruments are commonly used:—

“**PLATINUM LOOP.**”—This consists of a piece of platinum wire, No. 26 S.W.G., $2\frac{1}{2}$ in. long, with one end fused into a glass rod, or inserted into a special aluminium holder. The other end of the wire is bent in the form of a loop, care being taken that the loop is flat and completely closed. Owing to the high cost of platinum, “resistance” wire may be used as an efficient substitute.

The wire is sterilised by holding it vertically in a

Bunsen flame so that the whole length becomes red-hot at the same time. The platinum loop is the most useful of all the inoculating wires. Not only can a considerable amount of solid culture be taken up with it, but also a large drop of fluid.

STRAIGHT WIRE.—This is similar to the foregoing, but without the loop. It is used for stab cultures, and also for picking off single colonies.

LONG STRAIGHT WIRE.—A wire $4\frac{1}{2}$ in. long mounted on a holder. It is employed for deep-stab inoculation when working with anaerobes.

THICK WIRE, particularly with a loop, is very useful on account of its rigidity for lifting thick viscid sputum and tenacious growths.

WIRE WITH LANCE-HEAD.—This is made with thick wire (*e.g.* resistance wire), one end being flattened out and filed to a lance-head or diamond shape. It is employed for making scrapings from organs and for disintegrating felted cultures —*e.g.* fungi.

SCALPEL.—This instrument, sterilised by dipping in alcohol and flaming, is used for making inoculations with scrapings from tissues and ulcers, etc.

STERILE CAPILLARY PIPETTES.—These are made by heating the middle of a piece of quill tubing, 5 in. long, and when melted pulling out the two halves, thus forming two pipettes. The capillary ends, which should not be too thin, are sealed in the flame, and the other ends are plugged with cotton-wool. They are placed in a large test-tube $15 \times 2\frac{1}{2}$ in., which is then stoppered with cotton-wool and sterilised by dry heat. Before use, the end of the capillary portion is broken off and a rubber teat fitted to the other end. These pipettes are necessary for inoculating Smith-Noguchi medium and bullock heart medium (*q.v.*).

STERILE PIPETTES (10–100 c.c.) are used when large amounts of fluid inoculum have to be added to a medium.

Graduated pipettes are employed when measured quantities of material are used for inoculation (*vide* Water Examination).

TECHNIQUE OF INOCULATING TUBES

The following routine methods are employed by the writers and are recommended to beginners:—

Inoculation of one "slope" from another.—The two tubes are firmly held at their lower ends between the thumb and first two fingers of the left hand, with the sloped surface of the medium towards the worker. The tube containing the growth should be on the left and the uninoculated tube on the right. With the right hand loosen the cotton-wool stoppers by rotating them in the mouths of the tubes so that they may be removed easily. Take the holder of the inoculating wire at its end between the thumb and first two fingers of the right hand (as in holding a pen). Sterilise the wire by holding it vertically in the Bunsen flame. Remove the stopper of the tube from which the inoculation is to be made with the crooked third finger of the right hand, and flame the mouth of the tube. Pass the needle into the tube and touch a portion of the medium free from growth to ascertain if the needle is sufficiently cool. If too hot, the wire will melt the agar, causing a furrow, and would of course kill the organisms in removing the growth. When the wire is cool, the growth is scraped from the surface, care being taken not to wound the agar. Withdraw the wire, remove the stopper from the other tube with the crooked little finger and flame the mouth of the tube. Insert the wire charged with the growth and lightly smear the surface of the agar. Withdraw the wire and sterilise it, flame the mouths of the tubes and replace the stoppers. The nature of the inoculated material and also the date should be written on the tube by means of a grease pencil.

With *stab cultures*, the tubes are held similarly and the straight wire charged with bacterial growth is plunged into the centre of the medium, care being taken to withdraw the wire in the same line, and not to cause splitting of the medium.

In *inoculating a fluid medium*, such as broth, from

a solid culture, the tube should be inclined almost to the horizontal and the growth on the loop deposited on the wall of the tube just above the surface of the liquid at the lower end of the tube. On returning the tube to the vertical position the inoculum is now below the surface of the broth.

INOCULATING HOOD.—It is advisable, as far as possible, to carry out all inoculation procedures under a hood in order to minimise the chances of aerial contamination.

The hood employed by us has a wooden top and back, and two side windows. This is placed on the laboratory bench so that a chamber is formed, open only at the front. The top overhangs the bench by about 18 inches. The front is then closed by curtains made of calico, and the operator works completely under the hood. Ventilation is secured by two holes in the roof, covered with fine-mesh wire gauze. The table under the hood is covered by a towel soaked in 1 in 1000 perchloride of mercury solution, so that any organisms deposited in dust are destroyed. The advantage of the hood depends on the relative absence of dust and air currents, which are liable to produce contamination of medium, etc., exposed in the process of inoculation. We have used the inoculating hood with considerable success in the preparation of Smith-Noguchi medium, and in conducting autopsies on animals under aseptic conditions.

The bench on which the hood is fitted should have a gas supply for the Bunsen burner. The hood may be lighted by means of an electric lamp suspended from the top.

SEPARATION OF MIXED CULTURES

1. **BY SUCCESSIVE STROKES OR SPREADING.**—The platinum loop is charged with the bacterial mixture, pus, fragment of tissue, etc., and several strokes in series are made on the surface of the medium (either in a Petri dish or sloped in tubes) without recharging the wire.

An alternative method for Petri dishes is to employ a spreader. This is made by bending a piece of glass rod at a right angle in the blowpipe flame, the short limb, used for spreading, being 1 in. long. A small amount of the bacterial mixture is placed on the plate with the inoculating loop. By means of the spreader, previously sterilised by boiling and then cooled, the material is evenly distributed over the surface. The spreader is then transferred to a second plate, which is similarly inoculated. Thus the medium in the second dish is inoculated merely with the organisms carried over by the spreader from the first.

By these methods the bacteria are gradually wiped off the wire or spreader so that they are ultimately deposited singly. From each bacterium an isolated colony will grow; a single colony may be subcultured on fresh media and so yields a pure growth. *In order to ensure separation, the surface of the medium must be dry.*

2. BY PLATING¹ DECIMAL DILUTIONS OF THE INOCULUM.—A series of tubes of melted agar or gelatin are inoculated with successive decimal dilutions of the infected material and then poured into Petri dishes and allowed to solidify. By dilution, the bacteria are separated from one another, and on incubation the resulting colonies are distributed singly throughout the solid media, so that individual colonies may be removed and a pure culture obtained.

3. BY HEATING AND SUBSEQUENT PLATING.—This method is employed where the organisms to be obtained in pure culture are more resistant to heat than the remainder of the bacteria present. This method applies especially to spore-bearing organisms, such as the anaerobes, whose spores survive the heating. The mixture of bacteria is heated to 80° C. for half-an-hour and then plated. The spores form individual colonies, which may then be “picked off.”

¹ The term “plating” is also generally applied now to the inoculation of medium in Petri dishes by various methods—e.g. 1, *supra*.

Plate cultures should have the nature of the material, and also the date written on the glass of the Petri dish by means of a grease pencil. Agar plates are incubated in the inverted position—*i.e.* the lid of the plate is underneath and the grease-pencil writing should be on the portion of the dish containing the medium. On the other hand, gelatin, because it is liquefied by many organisms, is incubated with the lid uppermost, on which the necessary pencil notes are made.

Care must be taken in *picking off single colonies*, particularly when they are very close to one another, that the point of the needle does not touch any of the neighbouring colonies. The plate should first be looked at through the medium by holding it up to the light. The lid should be removed and the dish held round the side by the thumb and middle finger of the left hand. The colonies selected should be marked by grease-pencil rings on the bottom of the dish. To pick off the colony, first sit down with both elbows on the bench. Hold the plate vertically with the left hand, then grasp the holder of the wire like a pen, with the fingers quite close to the wire. Steady the right hand by placing the little finger on the left thumb in the way artists support the hand when painting. The selected colony is then easily removed without touching the others. Lay the plate on the bench, withdraw the right hand to the other end of the holder and inoculate the required medium in the manner previously described.

4. BY SHAKE CULTURE IN TUBES (*vide* p. 79).

5. BY ANIMAL INOCULATION.—Advantage is taken of the fact that laboratory animals are highly susceptible to certain organisms—for example, the mouse to the pneumococcus. If a mixture of organisms containing pneumococci—*e.g.* sputum—be inoculated into a mouse, the animal dies of pneumococcal septic-

aemia in twenty-four to thirty-six hours, and from the heart blood the pneumococcus can be obtained in pure culture. Similarly the tubercle bacillus can be isolated from contaminating organisms by inoculation of a guinea-pig. The tubercle bacilli are found in pure culture in the resulting lesions.

INCUBATION

Students and others commencing work in a laboratory should familiarise themselves with the mechanism of the incubator whereby any desired temperature may be constantly maintained. Incubators may be heated by electricity, gas or oil, according to the facilities of the laboratory. We have found electric incubators very reliable.

All bacteriological laboratories have at least one incubator working at 37° – 37.5° C. This temperature, which is the optimum for practically all pathogenic organisms, is the one referred to when speaking of incubation without mentioning the temperature.

Other temperatures for incubation are 30° C., used for cultivating leptospira, and 22° C. ("cool incubator"), used for certain fungi and for gelatin cultures.

Note.—Gelatin melts at 23° C.

Note.—In order to prevent drying of the medium where prolonged incubation is necessary, as in the cultivation of the tubercle bacillus, the mouths of the culture tubes are sealed with paraffin wax, or covered with special rubber caps.

METHODS OF ANAEROBIC CULTURE

Anaerobes are usually defined as organisms that will only grow in the absence of free oxygen. Anaerobic organisms, however, need oxygen for their metabolism just as much as any other living structure. They obtain it not direct from the air but from the breaking up of proteins and carbohydrates. Recent work has shown that it is not free oxygen by itself which is inimical to the growth of these organisms,

but that when molecular oxygen is present, a bacterial peroxide is formed, probably hydrogen peroxide, which prevents their multiplication. Anaerobes may be cultivated, therefore, either by preventing the admission of oxygen to cultures, or by destroying the peroxide as fast as it is formed, by means of catalase derived from fresh animal or vegetable tissue.

In the *Smith-Noguchi* method, for example, a combination of these methods is used. The cultures are sealed from the air by a vaseline plug; and any peroxide that may be formed is at once destroyed by the catalase present in a piece of fresh sterile rabbit kidney. This method is described on p. 73.

The method usually employed to establish anaerobic conditions is to remove the oxygen from the atmosphere surrounding the culture, the oxygen being sometimes replaced by an inert gas.

The simplest method of securing anaerobiosis is by growing the organisms in solid media. Deep agar tubes are convenient and efficient for the purpose. The addition of 1 per cent. glucose to the medium is of value, particularly when cultivating the saccharolytic group of anaerobes. Glucose acts as a reducing agent, thereby removing all oxygen from the medium, and further serves as a suitable pabulum for bacterial growth. The agar may be inoculated when solid by means of a long straight wire (*vide* p. 80). The colonies develop best in the depth of the tube, becoming fewer and smaller towards the surface. No grow this usually noted in the top half-inch of the medium. An alternative method is to melt the agar, cool it to 50° C. and introduce the inoculum by means of a capillary pipette. The contents of the tube are mixed by rotation between the palms of the hand. The agar is then rapidly solidified by placing the tube in cold water. The colonies develop in the deep portions of the tube usually separated from one another. This method is often employed in the preparation of pure cultures (see also p. 79).

Glucose broth can easily be rendered completely

anaerobic. Long tubes, $8 \times \frac{1}{2}$ in. (Noguchi tubes) are half filled with the medium and are placed in the steamer for half-an-hour or in boiling water for five minutes. Sterile melted vaseline is then poured on the surface of the medium and the tubes are rapidly cooled. The glucose and the heating remove all oxygen, and the vaseline effectively seals the medium from the air. Inoculation is made by means of a capillary pipette after melting the vaseline. Gas-producing anaerobes should not be cultivated in this medium, as the gas formed will force out the vaseline seal.

REMOVAL OF OXYGEN BY MEANS OF PYROGALLIC ACID AND CAUSTIC SODA

When pyrogalllic acid and caustic soda are mixed together, the mixture rapidly absorbs oxygen and becomes dark brown in colour. Many types of apparatus have been devised for the cultivation of anaerobes on plates, in which this method is used for absorbing oxygen. They are, however, inconvenient, and are not specially recommended.

For media in ordinary test-tubes, **BUCHNER'S METHOD** is applicable. The test-tube containing the medium is placed in a Buchner's tube, a stout-walled tube $8\frac{1}{2} \times 1$ in., with the lower end constricted so that the test-tube does not reach to the bottom of the tube. The tube is furnished with a well-fitting rubber bung. Some solid pyrogalllic acid is placed in the bottom of the tube, strong caustic soda is added, the inoculated tube is quickly introduced, and the rubber bung immediately inserted. The oxygen is rapidly absorbed and fairly satisfactory anaerobic conditions are obtained.

BULLOCH'S APPARATUS.—This consists of a special bell-jar with two openings at the top fitted with ground-glass stoppers. A glass tube with a stop-cock passes through each stopper and one of these tubes extends down inside the jar nearly to its base. The flanged rim of the jar rests on a thick ground-glass

plate, to which it is luted with resin ointment to secure an air-tight joint. Inside the jar, resting on the plate, is a large dish containing about 4 grams of pyrogallic acid crystals. The end of the long tube should be inside the dish. Plate cultures can be stacked inside the jar on a glass tripod.

Hydrogen is passed through the bell-jar to displace the air (*vide infra*) and then both stop-cocks are closed. The short glass tube is connected with an exhaust pump and a partial vacuum is created in the jar. A length of rubber tubing attached to the long glass tube dips into a beaker of strong caustic soda solution, and on opening the stop-cock the negative pressure in the jar draws the solution into the dish containing the pyrogallic acid. The stop-cock is then closed. The mixture of pyrogallic acid and caustic soda absorbs any oxygen that has not been displaced. The whole apparatus is then placed in the incubator.

M'INTOSH AND FILDES JAR

This apparatus, particularly Brown's modification, has given excellent results in our hands. It is easy to manipulate, and the degree of anaerobiosis is easily observed by means of an indicator inside the jar.

The principle of the apparatus is that spongy palladium and spongy platinum acting as catalytic agents cause the slow combination of hydrogen and oxygen to form water. The jar itself (12 × 5 in.) is made of stout glass, and has a tight-fitting lid that can be clamped down. The lid is furnished with a tube and tap, so that hydrogen may be introduced into the jar. Suspended from the lid by means of two wires, which are connected to terminals, is a small glass spool around which is the spongy palladium. This spongy palladium is made by immersing asbestos in a solution of palladium chloride and allowing it to dry; on heating in the blowpipe, the palladium is deposited in a black amorphous spongy layer on the asbestos. A fine coil of resistance wire is wound

round the palladiumised asbestos and the ends are connected to the two wires supporting the spool, so that a current can be passed through and the spongy palladium heated. The spool is surrounded by wire gauze, which, on the principle of the Davy lamp, prevents an explosion of the hydrogen and oxygen mixture.

Petri dishes or tubes are placed inside the jar, and also as an indicator a tube containing 10 c.c. of 2 per cent. glucose broth, to which 0.1 c.c. of a 1 per cent. aqueous solution of methylene blue has been added.

The lid is clamped down and the jar connected to a hydrogen supply. The current is turned on so that the palladiumised asbestos may be heated. The combination of oxygen and hydrogen takes place quietly in the jar. Water is formed, and more hydrogen enters to take the place of the oxygen consumed. After about twenty minutes all the oxygen is used up, and the tap is then turned off and the hydrogen supply disconnected. The jar is placed in the incubator, and within a few hours the indicator tube containing the methylene blue should be colourless, showing that complete anaerobiosis is established.

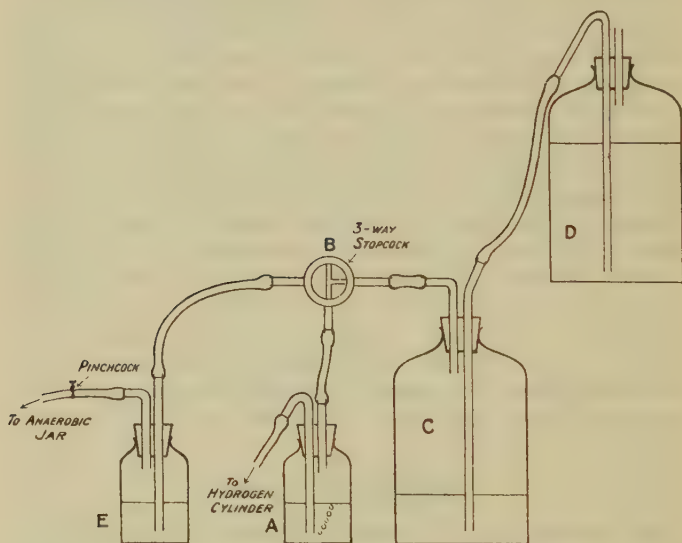
HYDROGEN SUPPLY

A hydrogen supply may be obtained from a Kipp's apparatus by the action of sulphuric acid on zinc. The gas must be purified by passing through three wash-bottles: (1) containing a solution of lead acetate, 1 in 10, to remove sulphuretted hydrogen; (2) containing a solution of silver nitrate, 1 in 10, to absorb arseniuretted hydrogen; and (3) containing a mixture of pyrogalllic acid and caustic soda, to remove oxygen.

It is more convenient, however, to obtain hydrogen from a cylinder containing the compressed gas. The commercial hydrogen so obtained is suitable for use in the various anaerobic apparatus employed. The hydrogen cylinder cannot be connected directly to the M'Intosh and Fildes jar, as the pressure is too

great. By the following apparatus, however, the amount of hydrogen entering the jar can be measured, and its pressure controlled.

Hydrogen from the cylinder is passed through the wash-bottle A, the rate of flow being estimated by the bubbles of gas rising through the water. From this the gas passes through the three-way stop-cock B to



a large jar C filled with water. This jar should have a capacity of 15–20 litres, and may be graduated in litres by adding measured amounts of water and marking the levels on a strip of $\frac{1}{2}$ -in. adhesive plaster attached to the jar. The jar is furnished with a rubber stopper and glass tubing as illustrated. The hydrogen entering C forces the water into a similar jar D, which is placed on a support four feet above C. When sufficient hydrogen has entered C, the cylinder supply is turned off. The stop-cock is then turned so that the gas can pass from C through the wash-bottle E, where the hydrogen flow can be estimated. As the

gas escapes from C, it is replaced by water siphoned over from D. The pressure of the hydrogen passing through E depends only on the height of the jar D above C.

Care must be taken to make the rubber stoppers in the jars and wash-bottles air-tight. This can be done by means of sealing-wax. Thick-walled "pressure" rubber tubing must be employed for all the connections.

DISPOSAL OF CULTURES

Cultures to be discarded should be killed by heat or antiseptics before the container is cleaned for re-use.

In the case of non-sporing organisms, it is sufficient to remove the cotton-wool plugs, and immerse the tubes and plugs in a large basin of 5 per cent. lysol or cresol.

Cultures of sporing organisms, such as B. tetani, B. anthracis, should be sterilised by autoclaving.

PERSONAL PRECAUTIONS IN BACTERIOLOGICAL LABORATORY WORK

It is essential to wear an overall while at work. If any material containing pathogenic organisms drops on the bench, floor, clothes, apparatus, etc., it should be sterilised at once with 1 in 1000 perchloride of mercury or 5 per cent. lysol. If the hands become contaminated they should be sterilised in a basin of lysol or perchloride of mercury solution, and workers should make it a rule to sterilise and wash the hands after completing any bacteriological work.

Precautions to be taken in carrying out special methods are referred to in the appropriate sections.

V

STAINING METHODS

As bacteria consist of clear protoplasmic matter, differing but slightly in refractive index from the medium in which they are grown, it is difficult, except by special methods of illumination, to see them in the unstained condition.

Staining, therefore, is of importance, not only for the recognition of bacteria, but also in virtue of the fact that by special methods certain organisms, such as tubercle bacilli and diphtheria bacilli, may be differentiated microscopically from others in mixtures.

Bacterial protoplasm reacts to stains in a manner similar to the nuclear material of tissue cells, and therefore the various basic aniline dyes are the commonest stains employed. The action of these stains may be intensified by the use of mordants such as phenol or weak alkalies, by the application of heat, and by prolonging the time of staining. Some organisms have a greater affinity for dyes than others, so that when stained and then treated with a decolorising agent they still retain the stain while others lose it. By such means it is possible to differentiate varieties of bacteria in a mixture.

METHODS OF MAKING FILM OR SMEAR PREPARATIONS

Before taking up the various staining processes, details of the methods employed in making films must be considered.

Film preparations are made either on the ordinary 3×1 in. glass slides or on cover-slips. It is essential

that the slides or cover-slips should be perfectly clean and free from grease, otherwise uneven films will result.

Cover-Slips.—These should be $\frac{7}{8}$ in. square, and of No. 1 thickness. Thicker cover-slips (No. 2) prevent the oil-immersion objective from coming near enough for the specimen to be focussed. They may be cleaned by placing them in a mixture of sulphuric acid, 6 parts; potassium bichromate, 6 parts; water, 100 parts. They should be dropped one by one into the fluid. The solution is contained in an evaporating dish and boiled. The cover-slips are then well washed in distilled water and stored in a stoppered jar in 50 per cent. spirit. Before use they are dried with a soft clean cloth, such as an old handkerchief.

Slides.—These may be treated in a manner similar to cover-slips. A quicker and quite satisfactory method for ordinary routine use is to moisten the finger with water, rub it on the surface of some fine sand soap such as "Monkey Brand" or "Vim," and then smear the surface of the slide. After removing the soapy film with a clean cloth the surface is clean and free from grease. If the slide is perfectly clean a drop of water can be spread over its surface in a thin even film, whereas if it is greasy the water collects into small drops and a film cannot be made.

In the case of fluid materials, such as broth cultures, urine, sputum, pus, etc., one or more loopfuls are taken up with the platinum wire and are spread evenly and thinly over the slide. A little experience will soon determine the amount required. The slide is then held in the palm of the hand, film downwards, over a Bunsen flame and dried. The film is fixed either by passing the slide three times slowly through the flame, or by heating through the glass slide. In the latter method the slide is held, film upwards, in the top of the Bunsen flame for a few seconds so that the slide becomes hot. Care must be taken not to char the film, and when the slide is just too hot to be borne on the back of the hand, fixation is complete.

Films on cover-slips require a minimum of time for fixing owing to the thinness of the glass. The cover-slips may be held conveniently by means of Cornet's forceps.

With solid material, such as cultures on agar, etc., it is necessary to place a loopful of clean water on the slide. The loop is then sterilised and a minute quantity of material, obtained by just touching the growth, is transferred to the drop, thoroughly emulsified, and the mixture is evenly spread on the slide. The resulting film is fixed and dried as above. Beginners are very apt to take more material than necessary from the culture and thus make too thick films.

STAINING

The method of staining varies with the nature of the preparation (film or section).

FILMS

The stains are poured directly or filtered on to the slide. When staining is completed, the dye is washed off with water and the slide placed between two sheets of white fluffless blotting-paper¹ or filter paper. The drying of the film is completed over the Bunsen flame. Such stained films may be mounted in Canada balsam under a cover-slip, or may be examined unmounted with the oil-immersion lens, a small drop of cedar-wood oil being placed directly on the film. If it is desired to mount the preparation later, the oil can be removed with benzol or xylol.

PARAFFIN SECTIONS

The sections being embedded in paraffin, it is necessary to remove the paraffin so that a watery stain may penetrate. The paraffin is first removed

¹ We have found Craig's "Velvet" blotting-paper suitable for the purpose.

with benzol or xylol, the benzol removed with alcohol, and the alcohol replaced by water. The staining process is then proceeded with. After staining, the section must be dehydrated with absolute alcohol, then cleared in benzol or xylol and finally mounted in Canada balsam under a cover-slip. The Canada balsam (which is a resin) is dissolved in xylol in order to render it of suitable consistence.

Technique.—The slide bearing the paraffin section is placed in a jar of benzol or xylol for some minutes to remove the paraffin. The section is then treated with a few drops of absolute alcohol, when it immediately becomes opaque. A few drops of 50 per cent. spirit are poured on, and the slide is finally washed gently in water. If the tissue has been fixed in any corrosive sublimate preparation, such as Zenker's fluid, the section should be treated with Gram's iodine solution for a few minutes (*vide* p. 120), then with methylated spirit and finally water. The sections are now ready to be stained. After staining and washing with water, the slide is wiped with a clean duster all round the section to remove excess of water. The bulk of the water in the section may be removed by pressing between fluffless blotting-paper. The section is *immediately* treated with a few drops of spirit, then absolute alcohol. The slide is again wiped all round the section, a few more drops of absolute alcohol are poured on, and the slide is then immersed in benzol or xylol. When cleared, a drop of Canada balsam is applied and the section mounted under a No. 1 cover-slip. It is essential that the section should not be allowed to dry at any period of the process, and that dehydration with absolute alcohol should be complete in order that the section may be thoroughly cleared.

SIMPLE STAINS

These show not only the presence of organisms, but also in exudates the nature of the cellular content.

METHYLENE BLUE

Of the many preparations of this dye, Loeffler's Methylene Blue is perhaps the most useful:

Saturated solution of methylene blue in alcohol	30 c.c.
Solution of caustic potash in water (1 : 10,000)	100 c.c.

(This caustic potash solution is conveniently made by adding 1 c.c. of a 1 per cent. solution to 99 c.c. of water.)

Films.—Stain for three minutes, then wash with water. This preparation does not readily over-stain.

Sections.—Stain for five minutes or longer. The application of the alcohol during dehydration is sufficient for differentiation. (Aniline oil can be used alternatively for dehydrating and clearing after the section is blotted.)

POLYCHROME METHYLENE BLUE

This is conveniently made by allowing Loeffler's Methylene Blue to "ripen" slowly. The stain is kept in bottles, which are half filled and shaken at intervals to aerate thoroughly the contents. The slow oxidation of the methylene blue forms a violet compound which gives the stain its polychrome properties. The ripening takes about twelve months to complete. The preparation is used in a manner similar to Loeffler's Methylene Blue, and is useful for the study of *Bacterium pneumosintes* (*vide* p. 284), and also for M'Fadyean's reaction (*vide* p. 194).

CARBOL THIONIN BLUE

Stock Solution:

Thionin blue	1 gram
Carbolic acid 1 in 40 watery solution	100 c.c.

For Use:

Stock solution	1 part
Distilled water	3 parts

(Filter before use.)

This method is useful for demonstrating such organisms as glanders and typhoid bacilli, in tissues.

- (1) Stain sections five to ten minutes.
- (2) Wash well with water.
- (3) Differentiate in a bowl of water to which a few drops of acetic acid have been added.
- (4) Wash well with water.
- (5) Blot, dehydrate with absolute alcohol, clear in benzol or xylol and mount in Canada balsam.

DILUTE CARBOL FUCHSIN

Made by diluting Ziehl-Neelsen's stain (p. 103) with ten to fifteen times its bulk of water. Stain for ten to twenty-five seconds and wash well with water. Over-staining must be avoided, as this is an intense stain, and prolonged application colours the cell protoplasm in addition to nuclei and bacteria.

GRAM'S STAIN

This is one of the most important stains in bacteriology, and is not only used for differentiating organisms, but must also be employed for diagnosis in the case of certain organisms, such as the gonococcus.

The principle of the stain is as follows:—certain bacteria when treated with one of the para-rosaniline dyes such as methyl violet or gentian violet, and then with iodine, fix the stain so that subsequent treatment with a decolorising agent—*e.g.* alcohol or aniline oil—does not remove the colour. Other organisms, however, are decolorised by this process. If a mixture of various organisms be thus stained and subjected to the decolorising agent, it will be found that some varieties retain the dye, and these are termed “Gram-positive,” whereas others are completely decolorised and are designated “Gram-negative.” In order to render the decolorised organisms evident, and to distinguish them from those retaining the colour, a contrast or counter-stain is then applied. This contrast stain is usually red, in order that the Gram-negative organisms

may be easily differentiated from the Gram-positive organisms, which retain the original violet stain. Gram's stain can only be carried out with the pararosaniline dyes—*e.g.* methyl violet, crystal violet, gentian violet (which is a mixture of the two preceding dyes) and victoria blue. Methyl violet and gentian violet are the usual stains employed.

WEIGERT'S MODIFICATION

Solutions employed—

(1) Carbol Gentian Violet:

Saturated alcoholic solution of gentian violet	1 part
5 per cent. solution of carbolic acid in distilled water	10 parts

(This mixture should be made up each day, as it tends to precipitate.)

(2) Gram's Iodine:

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	300 c.c.

(3) Aniline-Oil-Xylol:

Aniline oil	2 parts
Xylol	1 part

or [(4) Acetone-Alcohol:

Acetone	10 c.c.
Alcohol	100 c.c.]

(5) Dilute Carbol Fuchsin:

Ziehl - Neelsen's carbol fuchsin (p. 103)	1 part
Distilled water	9 parts

PROCEDURE

(a) *Films*.—The film is made, dried, and fixed in the usual manner.

(1) Stain film with carbol gentian violet (two to three minutes).

- (2) Pour off stain, replace with Gram's iodine solution, and allow to act for one minute.
- (3) Dry thoroughly by blotting with filter paper and gentle heat.
- (4) Decolorise with aniline-oil-xylol or acetone-alcohol solution, using several changes until the stain ceases to be removed.

Breathing on the slide after the first application of aniline oil hastens decolorisation.

Examine at this stage under the low power of the microscope; the nuclei of the pus cells should be of a pale violet colour; if the nuclei are intensely stained, then decolorising has not been carried sufficiently far.

- (5) Wash with several changes of xylol if aniline-oil-xylol is used, and allow to dry.
- (6) Counter-stain with dilute carbol fuchsin ten to twenty-five seconds. Wash with water and dry.

(b) *Sections*.—Counter-stain first with carmalum ten minutes, or dilute carbol fuchsin, 1 in 30, for one minute, and then proceed as above. If aniline-oil-xylol is used for decolorisation, the sections will also be cleared, and after (5) can at once be mounted in Canada balsam. If acetone-alcohol is used, the section must be dehydrated with absolute alcohol and cleared with xylol before being mounted.

JENSEN'S MODIFICATION

This method for smears and sections can be recommended particularly to those commencing staining methods.

Solutions required—

- (a) Methyl violet, 6 B., 0.5 per cent. solution in distilled water.

(The solution should be made up in bulk and filtered. It keeps indefinitely, does not precipitate, and does not need to be filtered again before use.)

(b) Iodine Solution :

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	100 c.c.

Note that the iodine solution is three times stronger than Gram's iodine.

(c) Counter-stain.—Neutral Red Solution :

Neutral Red	1 gram
1 per cent. acetic acid	2 c.c.
Distilled water	1000 c.c.

Film preparations.—Smears are made, dried, and fixed in the usual way.

- (1) Pour on methyl violet solution and allow to act for twenty to thirty seconds.
- (2) Pour off excess of stain, and, holding the slide at an angle downwards, pour on the iodine solution so that it washes away the methyl violet solution. Allow the iodine to act for a half to one minute.

Note.—It is important that the iodine should wash away the methyl violet, as any mixture of the two solutions causes a precipitate which deposits on the slide.

- (3) Wash off the iodine with methylated spirit, and treat with fresh spirit until colour ceases to come out of the preparation. This is easily seen by holding the slide against a white background.
- (4) Wash with water.
- (5) Apply counter-stain twenty to thirty seconds.
- (6) Wash with water and dry between blotting-paper.

This method is very simple, and gives excellent results with freedom from deposit.

Dilute carbol fuchsin (1:10) applied for twenty to thirty seconds may be substituted with advantage as a counter-stain for routine work, but for the diagnosis of gonococci, the neutral red counter-stain should be used.

Sections—

- (1) Dissolve out the paraffin in benzol or xylol.
- (2) Treat the section with absolute alcohol, then 50 per cent. spirit, and wash with water.
- (3) Pour on methyl violet solution and allow it to stain for one minute.
- (4) Replace the stain with iodine solution and allow it to act for one minute.
- (5) Replace the iodine with a few drops of spirit. Pour on clove oil and allow it to remain for about ten to fifteen seconds, washing the clove oil away with spirit after decolorising.

Note.—Clove oil is a very powerful decolorising agent and must be left on for only a short time. As it does not mix with water, the section is first treated with spirit, and when decolorisation is complete the oil is similarly removed.

- (6) Wash with water and apply counter-stain—thirty seconds if dilute carbol fuchsin, one minute if neutral red—and wash again with water.
- (7) Wipe the slide dry all round the section, and blot. Pour on a few drops of absolute alcohol, wipe the slide, treat with more absolute alcohol, wipe the slide again and place it in the benzol or xylol jar. The section is now dehydrated and cleared.
- (8) Mount in Canada balsam.

If Gram's stain is properly carried out, Gram-positive organisms and fibrin are stained dark violet in colour. Gram-negative organisms, the nuclei and protoplasm of pus cells and tissues cells are stained pink with the counter-stain.

To obviate errors from over-decolorising, a film of a known Gram-positive organism (*e.g.* a pure culture of *Staphylococcus aureus*) may be made at one side of the pus film. This "control spot" is stained along with the film. For the recognition of Gram-negative organisms, such as gonococci or meningococci in pus, the "control-spot" must retain the violet stain

while the nuclei of the pus cell are stained only with the counter-stain.

REACTION OF SOME ORGANISMS TO GRAM'S STAIN

Gram-Positive	Gram-Negative
Staphylococcus	Gonococcus
Streptococcus	Meningococcus
Pneumococcus	Micrococcus catarrhalis
Micrococcus tetragenus	Cocco-bacillus melitensis
Acne bacillus	Pneumobacillus of Friedländer
B. diphtheriae	Koch-Weeks bacillus
Hofmann's bacillus (pseudo-diphtheria bacillus)	Diplo-bacillus of Morax
B. xerosis	B. proteus
Tubercle bacillus. (This requires a special method, as the organ- ism is <i>not stained</i> by the ordinary Gram's method.)	B. pyocyaneus
Smegma bacillus	B. mallei
Leprosy bacillus	The coli-typhoid group, B. enteritidis Gaertner and allied organisms, and also the dysentery group of bacilli
Streptothrices	Vibrios— <i>e.g.</i> V. cholerae
B. anthracis	B. pestis
B. tetani	B. influenzae (Pfeiffer)
B. welchii	B. pertussis
B. sporogenes	B. fusiformis
Vibrio septique	Spirochaetes
B. botulinus	Bacterium pneumosintes

Note.—The above table refers only to young cultures, as degenerated specimens of organisms belonging to the Gram-positive class may react negatively.

STAINING FOR TUBERCLE AND OTHER ACID-FAST BACILLI

ZIEHL-NEELSEN METHOD

These organisms do not stain with the ordinary aniline dyes, on account of a resistant outer envelope

of a fatty nature which prevents penetration of the stain. The principle of staining these bacilli is the following :—

By the use of a powerful staining solution which contains a mordant, and the application of heat, the dye can be made to penetrate the organisms. As the organism is resistant to ordinary stains, it also tends to resist decolorisation. Once stained, the tubercle bacillus will withstand the action of powerful decolorising agents for a considerable time and thus still retains the stain when everything else has been decolorised.

The stain used consists of basic fuchsin, with phenol as the mordant. The dye is basic and its combination with an acid produces a compound which is light yellowish brown in colour. Any strong acid can be used as a decolorising agent, but sulphuric or nitric acid in a 20 per cent. solution is usually employed.

In order to show what has been decolorised, and to form a contrast with the red-stained bacilli, the preparation is counter-stained with methylene blue.

Ziehl-Neelsen's (strong) Carbol Fuchsin :

Basic fuchsin	1 part
Absolute alcohol	10 parts
Solution of carbolic acid (1:20)	100 parts

Dissolve the dye in the alcohol and add the solution to the carbolic acid.

Films.—Smears are made, dried and fixed in the usual manner :

- (1) Flood the slide with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for five minutes, heat being applied at intervals to keep the stain hot.
- (2) Wash with water.
- (3) Immerse the slide in 20 per cent. sulphuric acid. The red colour of the preparation is changed to yellowish brown. After about a minute in the acid remove the slide, wash with water and place it in the acid again. This process

should be repeated several times. The object of the washing is to remove the compound of acid and stain and allow fresh acid to gain access to the preparation. The decolorisation is finished when, after washing, the smear is a faint pink.

- (4) Wash the slide well in water.
- (5) Treat with methylated spirit for two minutes.
- (6) Wash with water.
- (7) Counter-stain with Loeffler's methylene blue for a half to one minute.
- (8) Wash, blot, dry and mount.

The bacilli stain bright red, while the tissue cells and other organisms are stained blue.

Note.—Other organisms are “acid-fast” in addition to the tubercle bacillus. The most important in diagnostic work is the smegma bacillus, which is frequently found in samples of urine. *This acid-fast organism is decolorised by alcohol, whereas the tubercle bacillus is not.* In other words, the tubercle bacillus is “acid-fast” and “alcohol-fast,” while the smegma bacillus and similar organisms are “acid-fast” but not “alcohol-fast.”

The decolorisation with spirit is particularly important when examining urine for the presence of the tubercle bacillus.

Sections.—

- (1) Sections are treated with benzol or xylol to remove paraffin, then with spirit, and finally washed in water.
- (2) Stain with Ziehl-Neelsen's stain as described for films, but heat gently, otherwise the section may become detached from the slide.
- (3) Wash with water.
- (4) Decolorise with 20 per cent. sulphuric acid as for films. The process takes longer owing to the thickness of the section, and care must be exercised in washing, to retain the section on the slide.
- (5) Wash well with water.
- (6) Counter-stain with methylene blue for one minute.

- (7) Wash with water.
- (8) Wipe the slide dry all round the section, blot with filter paper or fluffless blotting-paper, and treat with a few drops of absolute alcohol. Pour on more absolute alcohol, wipe the slide again and immerse it in the benzol or xylol jar.
- (9) Mount in Canada balsam.

Leprosy bacilli are also acid-fast, but not to the same extent. They are stained in smears or sections in the same way as the tubercle bacilli, except that 5 per cent. sulphuric acid is used for decolorisation.

STAINS FOR THE DIPHTHERIA BACILLUS

The diphtheria bacillus gives its characteristic staining reactions only in a young culture (twelve to eighteen hours) on serum medium (*vide* p. 182).

NEISSER'S STAIN

Solutions required—

- (1) Neisser's methylene blue.
- (2) (a) Cresoidin solution,
or (b) Bismarck-brown solution.
- (1) *Neisser's Methylene Blue*:

Methylene blue	1 gram
dissolved in absolute alcohol	20 c.c.
and added to glacial acetic acid	50 c.c.
in distilled water	950 c.c.
- (2) (a) Cresoidin 1 gram
 Distilled water 300 c.c.
 (Dissolve by gentle heat, and filter.)
- (b) Bismarck-brown 1 gram
 Distilled water 500 c.c.

The films are dried and fixed by heat.

- (1) Pour on Neisser's stain and allow to act for one minute.
- (2) Wash with water.

(3) Stain with Bismarck-brown or cresoidin for fifteen seconds.

(4) Wash with water and dry.

The protoplasm of the bacillus is brown, while the granules are blue-black in colour.

PUGH'S STAIN

Toluidin blue	2 grams
dissolved in absolute alcohol	20 c.c.
and added to glacial acetic acid	50 c.c.
in distilled water	950 c.c.

The dried film is flooded with stain and heated till steam rises. Stain for one to three minutes. The preparation will not over-stain. Wash with water and dry.

This is a very useful and rapid single stain.

The protoplasm of the bacilli is light blue and the granules are coloured reddish purple.

BISS'S STAIN FOR DIPHTHERIA BACILLI

This method has given excellent results in our hands for diagnostic purposes:

Methylene blue	2 grams
Dahlia	0.25 gram
Absolute alcohol	20 c.c.
Glacial acetic acid	50 c.c.
Distilled water	950 c.c.

The stains are dissolved in the alcohol, which is then added to the mixture of acetic acid and water.

In this method the specimen is examined mounted in the stain. A film is made and fixed on a *cover-glass*. The cover-glass is now mounted on a slide with a drop of the stain, in exactly the same way as a film on a cover-glass is mounted in Canada balsam. The excess of stain is removed by blotting so that the cover-glass does not float about on the slide. Examine the preparation at once under the oil-immersion lens. After a few seconds the diphtheria bacilli are seen with pale

blue protoplasm and deep blue granules, while other organisms are feebly stained. The preparation is not permanent, and fades in a short time.

STAINING OF SPORES

If spore-bearing organisms are stained by ordinary dyes, the body of the bacillus is deeply coloured, whereas the spore is unstained and appears as a clear area in the organism. This is because the spore has a resistant outer envelope which prevents the stain from penetrating the protoplasm in a manner similar to, but not to the same extent as, the tubercle bacillus (*q.v.*). Once the spores are stained, they retain the dye in spite of decolorising agents.

The following is a simple and satisfactory method for staining spores:—

Films, which must be thin, are made, dried and fixed in the usual manner.

- (1) Stain with Ziehl-Neelsen's carbol fuchsin for five minutes, heating the preparation until steam rises.
- (2) Wash in water.
- (3) Decolorise with
 - (a) $\frac{1}{4}$ or $\frac{1}{2}$ per cent. sulphuric acid,
 - or (b) methylated spirit.
- (4) Wash with water.
- (5) Counter-stain with Loeffler's methylene blue for two minutes.
- (6) Wash in water, blot and dry.

The spores are stained bright red and the protoplasm of the bacilli blue.

STAINING OF CAPSULES

Two methods are given. The first, Hiss's method, is useful for general routine work, and the second, Richard Muir's method, gives beautiful results, but is much more difficult for the beginner, and is not recommended for routine work.

HISS'S METHOD

Solutions required—

- (1) Saturated alcoholic solution of
 basic fuchsin 1 part
 Distilled water 19 parts
- (2) Copper sulphate solution 20 per
 cent. in distilled water.

Films should be thin and fixed by heat in the usual manner.

- (1) Pour on the stain and heat until steam rises.
 Allow the stain to act for thirty seconds.
- (2) Wash off the stain with the 20 per cent. copper
 sulphate solution, without washing in water,
 dry the film between blotting-paper, and
 mount, if necessary, in balsam.

In order to avoid an excessive deposit of copper sulphate crystals on the film, successive amounts of copper sulphate solution should be poured on the film until the slide is quite cool.

The bacteria are stained deep reddish brown, while the capsules are pale reddish brown in colour.

RICHARD MUIR'S METHOD

Solutions required—

- Strong carbol fuchsin.
 Special mordant.
 Loeffler's methylene blue.

Mordant :

- Saturated solution of corrosive
 sublimate 2 parts
 20 per cent. solution of tannic acid 2 parts
 Saturated solution of potassium
 alum 5 parts

The film, which must be very thin, is dried and fixed by heat.

- (1) Stain with strong carbol fuchsin for one minute,
 the preparation being gently heated.
- (2) Wash slightly with spirit and then well in water.

- (3) Pour on the mordant and allow it to act for thirty seconds.
- (4) Wash well with water.
- (5) Treat with methylated spirit. The time varies with the preparation and is found by trial. About thirty to forty seconds is ample, and the film should be pale red in appearance.
- (6) Wash well in water.
- (7) Counter-stain with Loeffler's methylene blue for a half to one minute.
- (8) The preparation may be washed and dried in the usual manner,
or dehydrated with absolute alcohol, cleared in xylol and mounted in balsam. This gives somewhat clearer specimens.

The bacteria are bright red, and the capsules are bright blue.

STAINING OF FLAGELLA

RICHARD MUIR'S MODIFICATION OF PITFIELD'S METHOD

This method, while yielding very good results, is often uncertain, and many trials may be necessary before a satisfactory preparation is attained. Success depends on rigorous cleanliness of cover-slips, a suitable culture, and freshly prepared solutions.

Solutions required—

(a) *Mordant:*

Tannic acid, 10 per cent. aqueous solution filtered	10 c.c.
Corrosive sublimate, saturated aqueous solution	5 c.c.
Potassium alum, saturated aqueous solution	5 c.c.
Ziehl-Neelsen's carbol fuchsin (p. 103)	5 c.c.

Mix thoroughly. A precipitate is formed, which is deposited by centrifuging or by allowing the fluid to stand. In either case

the clear supernatant fluid is removed to a clean bottle. The mordant keeps well for one or two weeks.

(b) *Stain* :

Potassium alum, saturated aqueous solution . . .	10 c.c.
Gentian violet, saturated alcoholic solution . . .	2 c.c.

This stain will not keep for more than two days.

Thin films are made from young agar cultures incubated about ten to sixteen hours. A small amount of the culture is emulsified in a little water in a watch-glass, the quantity of culture being only as much as will cause the faintest turbidity of the water. A film is made from a drop of the emulsion on a cover-slip, dried in the air and fixed by passing rapidly through the flame. The cover-slip should be held by means of Cornet's forceps.

- (1) Pour on the mordant and heat for one minute.
- (2) Wash well in running water for two minutes.
- (3) Dry the film carefully over the flame.
- (4) Pour on the stain and steam for one minute.
- (5) Wash well in water, dry and mount in Canada balsam.

THE ROMANOWSKY STAINS

The original Romanowsky stain was made by dissolving in methyl alcohol, the compound formed by the interaction of watery solutions of eosin and methylene blue. The original stain has now been replaced by various modifications which are easier to use and give better results ; these are :—Leishman's, Wright's, Jenner's and Giemsa's stains. The peculiar property of the Romanowsky stains is that they impart a reddish purple colour to the chromatin of malaria and other parasites. This colour is due to a substance which forms when methylene blue is "ripened" either by age, as in polychrome methylene

blue, or by heating with sodium carbonate. The latter method is employed in the manufacture of Leishman's and Wright's stains. The ripened methylene blue is then mixed with a solution of water-soluble eosin, when a precipitate, due to the combination of these dyes, is formed. The precipitate is washed with distilled water, dried, and dissolved in methyl alcohol which must be acetone-free. Each modification of the Romanowsky stain varies according to the "ripening" and the relative proportions of methylene blue and eosin.

According to the nature of the microscopic preparation, different stains are employed. Thus for the cytological examination of blood, Jenner's stain is used; for the malaria parasite and trypanosomes, Leishman's and Wright's modifications give the best results, while the pathogenic spirochaetes (particularly the *Spirochaeta pallidum* of syphilis) and certain protozoa can be demonstrated best by Giemsa's stain.

The Romanowsky stains are usually diluted, for staining purposes, with distilled water, when a precipitate is formed which is removed by subsequent washing.

JENNER'S STAIN

This can be purchased ready for use, but may be made by dissolving 0.5 gram of powdered stain (Grübler) in 100 c.c. acetone-free methyl alcohol. This stain is eminently satisfactory for the cytological examination of blood films, but is not so suitable for parasites.

The stain is poured on the dried but *unfixed* film and allowed to act for one minute. It is then diluted with twice its volume of distilled water, and the mixture is allowed to act for three minutes. The film is washed with distilled water and differentiated (the differentiation being controlled under the low power of the microscope) until the red blood corpuscles are pink. The slide is now blotted and allowed to dry in the air.

LEISHMAN'S STAIN

This stain may be purchased ready for use or made by dissolving 0.15 gram of Leishman's powder in 100 c.c. acetone-free methyl alcohol. The powder is ground in a mortar with a little methyl alcohol, the residue of undissolved stain allowed to settle and the fluid decanted into a bottle. The residue in the mortar is treated with more methyl alcohol, and the process is repeated until all the stain goes into solution. The remainder of the methyl alcohol is now added. The stain is improved by keeping two weeks before use.

Films.—Dry unfixed films are used. The stain is first used undiluted, and the methyl alcohol fixes the film. The stain is then diluted with distilled water, and the staining proper carried out.

- (1) Pour the undiluted stain on the unfixed film and allow it to act for thirty seconds.
- (2) By means of a pipette and rubber teat add double the volume of distilled water to the slide, mixing the fluids by alternately sucking them up in the pipette and expelling them. Allow the diluted stain to act for three minutes.
- (3) Wash the slide with distilled water, allowing the preparation to differentiate in the distilled water for half-a-minute.
- (4) Remove the excess of water with blotting-paper and dry in the air.

Sections—

- (1) The section is treated with benzol or xylol to remove the paraffin, then with alcohol, and finally distilled water.
- (2) Drain off the excess of water and stain for five to ten minutes with a mixture of 1 part stain and 2 parts of distilled water.
- (3) Wash with distilled water.
- (4) Differentiate with a weak solution of acetic acid (1:1500), controlling the differentiation under the low power of the microscope until

the protoplasm of the cells is pink and only the nuclei are blue.

- (5) Wash with distilled water.
- (6) Blot, dehydrate with a few drops of absolute alcohol, clear in xylol and mount in Canada balsam.

J. H. WRIGHT'S STAIN

This is similar to Leishman's stain and is more used in America than in this country. It should be purchased ready for use. The method of staining is, for all practical purposes, the same as for Leishman's stain.

GIEMSA'S STAIN

This consists of a number of compounds made by adding different proportions of methylene blue and eosin together. These compounds have been designated Azur I, Azur II, and Azur II-eosin. The preparation is best purchased ready made up by Grübler, but may be prepared as follows:—

Azur II-eosin	3 grams
Azur II	0.8 gram
Glycerin (chemically pure)	250 grams
Methyl alcohol (acetone-free)	250 grams

The stain may be used in a manner similar to Leishman's preparation (the "rapid method"), or prolonged staining may be carried out, as, for example, in staining spirochaetes (the "slow method"). In both cases the preparation must be fixed prior to staining, either by methyl alcohol for three minutes, or by absolute alcohol for fifteen minutes.

RAPID METHOD

- (1) Fix films in methyl alcohol—three minutes.
- (2) Pour on a mixture of 1 part stain and 2 parts distilled water, and allow to act for five minutes.
- (3) Wash with distilled water, allowing the preparation to differentiate for about half-a-minute.
- (4) Blot and allow to dry in the air.

SLOW METHOD

A specially useful method for demonstrating objects difficult to stain in the ordinary way, such as the pathogenic spirochaetes. The principle is to allow the diluted stain to act for a considerable period. As the mixture of stain and water causes a fine precipitate, care has to be taken that this does not deposit on the film.

Cover-slips.—The film is fixed in methyl alcohol for three minutes. A mixture is made in a Petri dish in the proportion of 1 drop of stain to 1 c.c. of distilled water. The cover-slip, when fixed and still wet with the alcohol, is placed carefully, film downwards, on the surface of the mixture. When properly done, the cover-slip remains floating. The lid is carefully placed on the Petri dish and the stain allowed to act overnight. The cover-slip is then washed in a stream of distilled water, allowed to dry in the air and mounted. There should be no deposit of stain precipitate on the preparation.

Slides.—The film is fixed in methyl alcohol for three minutes as with cover-slips. The mixture of stain and distilled water is made in a large (6 in.) Petri dish if there are several slides to stain. A piece of thin glass rod is placed in the Petri dish and the slides, after fixing, are laid, film downwards in the fluid, with one end of the slide resting on the glass rod so that there is sufficient staining fluid between the film and the bottom of the dish. After sixteen to twenty-four hours staining, the slides are washed and dried as in the case of cover-slips.

BORREL'S BLUE EOSIN MIXTURE

This preparation gives staining results similar to the Romanowsky stains.

Borrel's Blue.—Dissolve 10 grams of silver nitrate

in 50 c.c. of distilled water in a flask of 150 c.c. capacity. Add 50 c.c. of 10 per cent. NaOH, when a precipitate, at first white and later becoming brown, is observed. Shake the flask and filter. The precipitate (silver oxide) is washed with distilled water until free from alkali. It is then returned to the flask, which is filled three-quarters full with a saturated watery solution of methylene blue. After allowing the mixture to mature for one month it is filtered.

Borrel's Blue Eosin :

Borrel's blue	1 c.c.
Eosin 0.1 per cent.	5 c.c.
Distilled water	4 c.c.

- (1) Fix the film in methyl alcohol for three minutes.
- (2) Wash in distilled water.
- (3) Stain in Borrel's blue eosin for fifteen minutes.
- (4) Wash in distilled water.
- (5) Treat with 5 per cent. tannic acid two minutes.
- (6) Wash in distilled water, blot and dry in the air.

FONTANA'S METHOD FOR DEMONSTRATING SPIROCHAETES

Solutions required—

(a) *Fixative :*

Acetic acid	.	.	.	1 c.c.
Formalin	.	.	.	20 c.c.
Distilled water	.	.	.	100 c.c.

(b) *Mordant :*

Carbolic acid	.	.	.	1 gram
Tannic acid	.	.	.	5 grams
Distilled water	.	.	.	100 c.c.

(c) *Ammoniacal silver nitrate :*

Add 10 per cent. ammonia to $1\frac{1}{2}$ per cent. solution of silver nitrate in distilled water until the precipitate formed just dissolves. Now add more silver nitrate solution drop by drop until the precipitate returns and does not re-dissolve.

Method—

- (1) Treat the film three times, thirty seconds each time, with the fixative.
- (2) Wash off the fixative with absolute alcohol and allow alcohol to act for three minutes.
- (3) Drain off the excess of alcohol and burn off the remainder carefully until the film is dry.
- (4) Pour on the mordant, heating till steam rises, and allow it to act for half-a-minute.
- (5) Wash well in distilled water and again dry the slide.
- (6) Treat with ammoniacal silver nitrate, heating till steam rises, for half-a-minute, when the film becomes brown in colour.
- (7) Wash well in distilled water, dry and mount in balsam.

It is essential that the specimen be mounted under a cover-slip before examination, as some immersion-oils cause the film to fade at once.

The spirochaetes are stained brownish black on a brownish yellow background.

LEVADITI'S METHOD OF STAINING SPIROCHAETES IN SECTIONS

(a) ORIGINAL METHOD

1. Fix the tissue, which must be in small thin pieces (1 mm. thick), in 10 per cent. formalin for twenty-four hours.

2. Wash the tissue for one hour in water and thereafter place it in 96–98 per cent. alcohol for twenty-four hours.

3. Transfer to 1.5 per cent. silver nitrate solution in a dark bottle and keep in the incubator for three days.

4. After washing in water for twenty to thirty minutes, place the pieces of tissue in the reducing mixture, made up as follows:—

Pyrogallic acid	4 grams
Formalin	5 c.c.
Water	100 c.c.

and allow them to remain in this mixture in a dark bottle for forty-eight hours at room temperature.

5. After washing well with water, dehydrate the tissue with increasing strengths of alcohol and embed in paraffin (see p. 121). Thin sections are cut and mounted in the usual way. After removing the paraffin with benzol or xylol the sections are straight-way mounted in Canada balsam.

(b) NEWER PYRIDIN METHOD

This method is more rapid, but in our experience does not give quite so good results as the older method:

1. Fix the tissue in formalin as in the other method, harden in alcohol overnight and then wash in distilled water.

2. Place the tissue in a 1 per cent. solution of silver nitrate (to which one-tenth of the volume of pure pyridin has been added) for two hours at room temperature, and thereafter at about 50° C. for four to six hours. It is then rapidly washed in 10 per cent. pyridin solution.

3. Transfer to the reducing fluid, which consists of

Formalin 4 per cent. . . . 100 parts

to which are added immediately before use

Acetone (pure) 10 parts

Pyridin (pure) 15 parts

Keep the tissue in this fluid for two days at room temperature in the dark.

4. After thorough washing, the tissue is dehydrated, embedded, cut and mounted in the usual way.

STAINING OF AMOEBAE AND OTHER INTESTINAL PROTOZOA IN FAECES

Wet smears should be fixed in a mixture of

Alcohol	1 part
Saturated aqueous solution of corrosive sublimate	2 parts

for twelve to twenty-four hours.

The films are then washed thoroughly in water to remove the corrosive sublimate, or may be treated with Gram's iodine for two minutes, the iodine being removed with spirit and the films washed.

Stain with iron haematoxylin for ten to twenty minutes.

Iron Haematoxylin:

(a) Haematoxylin	1 gram
Absolute alcohol	100 c.c.
(b) Liquor ferri. perchlor. 30 per cent.	4 c.c.
Concentrated hydrochloric acid	1 c.c.
Distilled water	100 c.c.

Mix equal parts of (a) and (b) immediately before using.

After staining, wash films in water.

Preparations may be counter-stained with Van Gieson's stain, fifteen to thirty seconds:

Saturated aqueous solution of acid fuchsin	1-3 parts
Saturated aqueous solution of picric acid	100 parts

Dehydrate rapidly with absolute alcohol, clear in benzol and mount in balsam.

Note.—Fixed wet preparations must be treated in the same manner as sections and never allowed to become dry.

FIXATION AND EMBEDDING OF TISSUES; SECTION CUTTING

As the ordinary routine bacteriological investigation of tissues is carried out almost exclusively with paraffin sections, this technique only will be described.

The tissue is embedded in paraffin wax to support it during the cutting of the section, and the section is held together by the wax in the process of transferring it to the slide.

The paraffin wax must completely permeate the tissue, but before it can do so all water must be removed from the material and replaced by a fluid with which melted paraffin will mix.

Water, therefore, is first removed by alcohol; the alcohol is replaced by some fluid, such as benzol, xylol, acetone, chloroform, which is a solvent of both alcohol and paraffin wax, and the tissue is finally embedded in melted paraffin.

Before removing the water from the tissue preparatory to embedding, the tissue must be suitably fixed and hardened.

The essentials for obtaining good sections are :

- (1) The tissue must be perfectly fresh.
- (2) It must be properly fixed by using small pieces and employing a large amount of fixing fluid.
- (3) The appropriate fixing fluid must be employed for the particular investigation required.
- (4) The tissue must not remain too long in the embedding bath.

FIXATIVES

FORMALIN

Ten per cent. commercial formalin in normal saline solution is a good fixative for general use. Its advantages are : it is easily prepared, has good penetrating qualities, does not shrink the tissues, and permits considerable latitude in the time during which specimens may be left in it. Moreover the subsequent

handling of the material is much easier in our experience than in the case of corrosive sublimate fixatives, such as Zenker's fluid. Formalin fixation is not so good as other methods where fine detail has to be observed, as, for example, in material containing protozoa. For general routine use, however, it is the most convenient and useful of fixatives. Tissue should be cut into thin slices, about $\frac{1}{8}$ -in. thick, and dropped into a large bulk of fixative. The fluid may be changed at the end of twenty-four hours, and fixation is usually complete in forty-eight hours. Specimens are then washed in running water for an hour and transferred to 50 per cent. spirit. In the latter fluid they may be kept for a considerable time without deterioration.

ZENKER'S FLUID

Potassium bichromate	.	.	2.5 grams
Corrosive sublimate	.	.	5-8 grams
Water	.	.	100 c.c.

Immediately before use, 5 c.c. of glacial acetic acid per 100 c.c. of fluid are added.

The fluid should be warmed to body temperature and only small pieces of tissue must be placed in it. Fixation is complete in twenty-four hours, and thereafter the pieces of tissue are washed in running water for twenty-four hours to remove the potassium bichromate and corrosive sublimate. The tissue is then transferred to 50 per cent. spirit.

It is essential that all corrosive sublimate should be removed, otherwise a deposit will appear in the sections. The bulk of the corrosive sublimate is removed by washing. The remainder can be removed with iodine after the sections are cut and mounted, and are ready for staining; Gram's iodine is applied to the section, allowed to act for two minutes, and then removed with spirit.

In our experience, animal tissues fixed in Zenker's fluid are more difficult to cut, and sections are apt to float off the slide.

EMBEDDING AND SECTION CUTTING

After fixation by either method and transference to 50 per cent. alcohol, pieces of tissue are treated as follows :—

- (1) Place in 90 per cent. spirit for twenty-four hours.
- (2) Transfer to absolute alcohol for twenty-four hours.
- (3) Complete dehydration in fresh absolute alcohol for twenty-four hours.
- (4) Transfer to a mixture of absolute alcohol and chloroform (equal parts) for twenty-four hours.
- (5) Place in pure chloroform for twenty-four hours.
- (6) Transfer the tissue for twenty-four hours to a mixture of equal parts of chloroform and paraffin wax, which is kept melted in the paraffin oven.
- (7) Place in pure melted paraffin in the oven at 55° C. for twenty-four hours, preferably in a vacuum embedding oven.

Note.—If necessary this process can be expedited by using very small pieces of tissue and by shortening the periods of time in the various fluids.

The tissue is embedded in blocks of paraffin. These are cut out, trimmed with a knife, and sections of 5 μ thick cut by means of a microtome. The sections are flattened on warm water, floated on to slides and allowed to dry. Albumenised slides are useful where the staining process involves heating.

For further treatment of sections, see Staining Methods.

For additional details, reference must be made to works on histology.

VI

ANIMAL INOCULATION

IN the British Isles animal experiments may only be done under a licence granted by the Home Secretary. In addition to the licence various certificates have also to be obtained, depending on the nature of the experiments and the animals used.

The usual animals employed for bacteriological experiments are the guinea-pig, rabbit, mouse and rat, and *the commonest method of inoculation is by means of a hypodermic needle and syringe*. According to the method of inoculation and the size of the animal, the amount of injected material varies, and a number of syringes of different sizes are used. A convenient "battery" of syringes is the following:—1 c.c. "tuberculin" syringe graduated in 0·01 c.c.; 2 c.c. syringe graduated in 0·1 c.c.; 5 c.c. graduated in 0·25 c.c.; 10 c.c. graduated in 0·5 cc. A 20 c.c. syringe is only occasionally used. Syringes may be of the "Record" type or of the all-glass "Luer" type. A selection of needles is needed, of which the following are useful:—fine bore, No. 25 gauge; medium bore for general use, No. 21 or 22 gauge; large bore, Nos. 16–18 gauge, for inoculating thick suspensions or emulsions of tissue.

Syringes are sterilised by taking them apart and boiling for five minutes. After use they should be washed out with weak lysol and again boiled. They are then dried and put away with the piston and barrel separate. Needles may be stored in alcohol, or dried and a little alcohol run through them. The stilette should be lightly smeared with vaseline before replacing it in the needle. Needles should always

be kept sharp and the points renewed on a fine oil-stone.

MATERIAL INOCULATED

EXUDATES such as empyema fluid, urine, cerebro-spinal fluid and blood are easily inoculated with a medium bore needle. Tenacious exudates such as pus and sputum are injected by means of a wide bore needle.

CULTURES.—Fluid cultures are easily drawn through a medium bore needle. It will be found advantageous first to pour the culture into a small (2 in.) Petri dish, or a 50 c.c. conical test-glass. Growths on solid media may be scraped off and emulsified in broth or saline, or the diluting fluid may be poured on the culture, which is then emulsified with a platinum needle.

TISSUES.—These should be cut into small pieces in a porcelain mortar by means of scissors sterilised by boiling. Some clean coarse sand, contained in a stoppered bottle, and sterilised by hot air, is then added to the mortar and the whole thoroughly ground with the pestle. When the tissue has been well ground up, saline is added, and the mixture further triturated. On standing for a short time, the sand and tissue rapidly settle to the bottom of the mortar, and the supernatant fluid can be drawn into the syringe. When intravenous inoculation of tissue emulsion has to be employed, care must be taken that no solid particles are injected. To avoid this, the emulsion must be centrifuged at low speed, and only the supernatant fluid used.

GUINEA-PIGS

These animals vary in size, and weigh from 200 grams (small) to 1000 grams (large). A good average weight for general purposes is 400 grams. Guinea-pigs are used in the diagnosis of the following diseases :—

Tuberculosis, diphtheria, anthrax, glanders, plague,

spirochaetal jaundice, typhus fever, gas gangrene, botulism.

Details of materials inoculated and methods are given in the appropriate sections.

METHODS OF INOCULATION

Subcutaneous.—An assistant holds the animal during the operation, and the injection is made under the skin of the flank. The animal is grasped across the shoulders in one hand, with the thumb curved round the animal's neck so that it rests on the lower jaw. The hind legs are secured between the first and second, and second and third fingers of the other hand, the knuckles being uppermost, and the animal is held so that the flank is presented for inoculation. The skin may be disinfected with tincture of iodine. The operator picks up a fold of skin and introduces the point of the needle into the base of the fold, so that it lies in the subcutaneous tissue. Amounts up to 3 c.c. can be introduced. A 2 c.c. or a 5 c.c. syringe is convenient for the purpose.

Intraperitoneal.—The animal is held in a similar manner. The inoculation is made in the mid-line in the lower half of the abdomen. The skin may be painted with iodine. The assistant holds the animal with its head downwards, so that the intestines fall towards the diaphragm. The skin is pinched up, the point of the needle passed into the subcutaneous tissue, and then downwards through the abdominal wall into the peritoneal cavity. The risk of damage to the intestines is practically nil. Not more than 5 c.c. can safely be inoculated intraperitoneally.

RABBITS

These animals are often unsatisfactory for experimental purposes owing to their liability to parasitic and intercurrent infections. The animals used should be free from snuffles (a chronic nasal inflammation),

subcutaneous abscesses, and mange. They should be plump, their fur should be in good condition, and they should not be suffering from diarrhoea. If the animal is in poor condition it is probably affected with coccidiosis or intestinal worms. Rabbits are very prone to die from septicaemia (due to *B. leptosepticus*), and from pneumonia. It is better to use animals obtained from a reliable breeder where the condition of the stock is known, than to purchase rabbits casually and indiscriminately from a dealer.

The normal rectal temperature of the rabbit is 102.4°F. , but the variations are great. No temperature under 104°F. should be considered pathological. The leucocyte count of the rabbit is subject to great normal variation.

Rabbits are used in the diagnosis of the following diseases :—

Tuberculosis.—Determination of the type of bacillus. A small amount of culture— $0.1-0.01$ mgm.—is inoculated intravenously (*vide* p. 186).

Rabies.—Intracerebral inoculation of brain tissue from suspected dog (done under ether anaesthesia) (*vide* p. 287).

The chief use of the rabbit lies not so much in diagnostic work as in its value for experimental purposes. It is extensively used for the production of immune sera, such as agglutinating and haemolytic sera, which are frequently employed for routine laboratory diagnosis.

Under Certificate "A" of the Home Office the animal may be inoculated intravenously, intraperitoneally or subcutaneously, without the use of an anaesthetic.

Intravenous inoculation is employed when material has to be introduced directly into the circulation. The marginal vein of the ear is the most convenient site. The rabbit may be held by an assistant or placed in a special box so that only its head protrudes. The hair over the vein should be dry-shaved with a sharp razor. The vein may be distended for ease of inocula-

tion either by vigorous rubbing with a piece of cotton-wool or by holding the ear over an electric-light globe, when the heat causes a dilatation of the blood vessels. According to the amount of material to be injected, a suitable syringe furnished with a needle is selected and sterilised by boiling. It is a mistake to use too fine a needle. The operator faces the animal and the ear is held horizontal by means of the left hand. The needle is kept as parallel as possible to the vein and the point inserted towards the head of the animal. When the injection is completed, the needle is withdrawn, and a small piece of cotton-wool placed on the vein, which is then compressed between the thumb and finger.

Note.—In removing samples of blood from the rabbit a similar procedure is adopted. The ear is shaved, sterilised with alcohol and ether, the vein dilated, and an incision is made into it by means of a large Hagedorn needle or a sharp scalpel. The blood is then allowed to drop into a test-tube.

Intraperitoneal inoculation is carried out as in the case of the guinea-pig.

Subcutaneous inoculation may be made either into the abdominal wall, or into the loose tissue about the flank or at the back of the neck. The hair is clipped, the skin is sterilised with iodine and then pinched up, and the needle is inserted. The technique is the same as that for the guinea-pig.

RATS

Care must be exercised in handling these animals, as the sharp incisor teeth are capable of inflicting a severe wound, and there is always a possibility of rat-bite fever ensuing (*vide* p. 263). They should be held by the loose tissue at the nape of the neck with a pair of crucible tongs, and the animal is kept taut by pulling on the tail. Intraperitoneal and subcutaneous injections are made in a manner similar to that used for the guinea-pig.

MICE

White mice are usually employed. They are extremely susceptible to the pneumococcus and are extensively used in the technique of typing the various strains of this organism (*vide* p. 170). Intraperitoneal or subcutaneous inoculation of even extremely small doses of the pneumococcus causes death from septicæmia in twenty-four to thirty-six hours. From the heart blood the organism can be recovered in pure culture.

Subcutaneous inoculation.—An assistant grasps the loose skin at the nape of the neck in one hand and the tail in the other. In this manner the animal is held in a fixed position while the needle is introduced under the skin near the root of the tail. Amounts up to 1 c.c. may be injected.

Intraperitoneal inoculation may be carried out if the animal is similarly held and then turned over. For steadiness, the assistant's arms should rest on the table. The injection is made to one side of the middle line in the lower half of the abdomen and amounts up to 2 c.c. can be introduced.

Intraperitoneal inoculation may also be done without an assistant. The animal is held at the nape of the neck with the left hand, and kept extended by holding the tail with the right hand. The left hand is turned over so that the mouse lies on its back in the upturned palm. The tail is then fixed by the little finger of the left hand. The mouse is now firmly held, and the right hand is free to pick up the syringe and make the injection.

AUTOPSY

All experimental animals, whatever the cause of death, should be autopsied as a routine. When a virulent organism such as the bacillus of plague or of anthrax has been used, especial care must be taken, otherwise the infection may be disseminated with danger to the operator and other workers.

Details will be given of the procedure in conducting an autopsy in the usual manner, and also the method used when dealing with highly infectious organisms.

As the primary reason for the autopsy is to recover organisms previously injected into the animal, the examination must be conducted with strict aseptic precautions.

Materials required :

A suitable animal board, on which the carcase can be fixed in the supine position.

Instruments.—Three scalpels ; scissors, ordinary size, four pairs ; mouse-toothed forceps, four pairs ; small bone forceps if the skull is to be opened ; a searing iron—a 4 oz. soldering bolt is excellent for the purpose ; sterile capillary pipettes ; sterile Petri dishes ; sterile test-tubes, and tubes of media.

The knives are sterilised in strong lysol, and the metal instruments by boiling in a sterilising bath—*e.g.* an enamelled “fish-kettle.” When ready for use, the tray of instruments is lifted out of the steriliser and laid on a spread towel which has previously been soaked in 1 in 1000 solution of perchloride of mercury.

It is our practice, where cultures have to be made, first to immerse the animal completely in weak lysol solution (5 per cent.) for ten minutes. This not only destroys most of the surface organisms, but prevents the dust in the fur from getting into the air and causing contamination. The animal is now fixed to the board, and towels moistened with antiseptic are placed over the head and lower extremities.

The instruments are removed from the steriliser. A long median incision through the skin of the abdomen and chest is now made, and the skin widely dissected, exposing the abdominal and chest muscles. Using another set of instruments the peritoneal cavity is opened, and the abdominal wall is reflected to each side. With fresh instruments remove the spleen and place it in a sterile Petri dish. Other organs such as the liver and kidneys may be similarly removed. The

ensiform cartilage is now tightly gripped with a pair of strong forceps, and by means of a sterile pair of strong scissors a cut is made on either side of the chest through the costal cartilages. The sternum is raised and pulled towards the head. The heart is now exposed. A sterile capillary pipette, furnished with a teat, is passed through the heart wall. Blood can thus be withdrawn and inoculated into various media. If the autopsy has been properly performed, it is not necessary to sear the surface of the heart. The lungs are then removed, using fresh instruments, by cutting the organs free at the hilum. Care must be taken not to open into the oesophagus if the lungs are to be used for cultural purposes.

After the organs to be used for culture have been removed and placed in separate Petri dishes, the autopsy can be completed.

While the instruments are again being boiled the naked-eye appearances of the organs should be studied. For culture, the spleen gives the best results, but the other solid viscera may be similarly used. The organ is cut with sterile instruments and a small portion is taken up with a stiff wire and smeared on the surface of solid media. Liquid media are inoculated with a small fragment of the tissue.

In conducting a post-mortem examination, various animal diseases, such as worms, coccidiosis, pseudo-tuberculosis, etc., may be noticed, and the worker should be familiar with their appearances.

When the animal is infected with highly pathogenic organisms, the first duty of the worker is to wear rubber gloves. The animal is soaked in antiseptic solution as before, and nailed to a rough piece of board of the appropriate size. This board is then placed in a large enamelled tray. The autopsy is carefully performed in the usual way. The whole carcase is finally covered with 20 per cent. lysol, which flows over the board and into the tray. The whole contents of the tray—board and carcase—are then destroyed in the furnace. The rubber gloves, instruments and tray

are thoroughly sterilised. When performing animal autopsies we strongly advise the wearing of a large overall made of waterproof material, and in addition, the wearing of some form of glasses or goggles to protect the eyes.

VII

SPECIAL BACTERIOLOGICAL METHODS

BLOOD CULTURE

Requisites :

- (1) A 10 c.c. syringe sterilised by boiling in water ; the syringe must not come into contact with antiseptic ; it should not be removed from the steriliser until it is immediately required, and the parts should be taken out of the steriliser and fitted together with the aid of forceps so that the needle, nozzle and piston are not touched by the fingers.
- (2) Gauze or cotton-wool, bandage, antiseptic (4 per cent. iodine in spirit or 1 : 20 carbolic acid), methylated spirit, collodion, dissecting forceps, Bunsen burner or spirit lamp.
- (3) 100 c.c. sterile bouillon in a stoppered flask.

The blood is drawn by vein puncture. The skin of the patient's arm at the bend of the elbow is *thoroughly sterilised*, particularly to obviate contamination of the culture with skin organisms—*e.g.* staphylococcus albus. Several turns of a bandage are applied round the upper arm about the middle of the biceps to render the veins turgid, or a piece of rubber tubing firmly, but not too tightly, wound once round the arm and clipped with pressure forceps provides a convenient and easily released tourniquet for the purpose. The turgescence of the veins can be increased by the patient's alternately opening and clenching the hand. The needle (attached to the syringe) is inserted into a prominent vein and 5–10 c.c. of blood are drawn into the syringe. The

tourniquet is then released. The needle is now withdrawn from the vein and detached from the syringe by means of forceps so that the nozzle is not touched by the fingers. The flask of bouillon is unstoppered and the mouth of the flask flamed. The blood is added to the bouillon and the flask re-stoppered. The blood and broth are thoroughly mixed by rotation of the flask. The flask is incubated at 37.5°C .

The syringe and needle should be at once washed out with water. The puncture wound is dressed with gauze or cotton-wool, and collodion.

When the flask has to be transported some distance to the laboratory it is advisable to stopper it with a sterile rubber cork sterilised by boiling, and inserted into the flask with flamed forceps.

In suspected cases of enteric fever, instead of bouillon as the culture medium, sterilised ox bile or sterile 0.5 per cent. sodium taurocholate may be used. Five c.c. of blood are added to 10 c.c. of bile or to 50 c.c. of taurocholate solution.

After incubation for eighteen to twenty-four hours, films are made from the bouillon and stained by Gram's method. If organisms are noted, sub-inoculations are made on nutrient agar slopes or an agar plate by the successive stroke method (*vide* p. 82). The sub-cultures are incubated and the organisms developing are identified as far as possible by their microscopic characters and colony appearances. If further investigations are required for accurate identification, single colonies are "picked off" on to agar slopes and the resulting cultures are studied.

Where an infection with the enteric group is suspected, it is convenient to plate direct from the blood culture on MacConkey's medium to elicit the characteristic colonies on this medium.

Even when no organisms can be detected in films from the primary blood culture, it is advisable to make sub-inoculations, as scanty organisms may not be observed, but still develop colonies in subculture.

If no result is obtained after twenty-four hours incubation, the blood bouillon should be incubated continuously for *at least* four days, films and sub-inoculations being made each day.

THE WIDAL REACTION AND OTHER AGGLUTINATION TESTS

The nature of the *Widal reaction* and its applications are referred to on pp. 28, 208.

DIRECTIONS FOR OBTAINING SPECIMEN OF BLOOD SERUM FOR THE TEST

The blood specimen may be taken, as for the Wassermann reaction, by vein puncture (*vide* p. 140), but generally a small amount of serum suffices for the Widal test, and this can easily be obtained as follows :—

The *requisites* are—straight Hagedorn needle, small glass-cutting file, spirit lamp or Bunsen burner, gauze or cotton-wool, methylated spirit, and a Wright's blood capsule (1½–2 in. of plain glass quill tube No. 5, drawn out at the ends to capillary dimensions and sealed, with one of the capillary limbs bent in a semicircle).

The blood is drawn by puncturing the skin of the finger near the base of the nail. Cleanse the needle and the patient's finger with spirit. Break off the tips of both extremities of the capsule, notching first with the file. Puncture the skin by a deep thrust of the needle. When a large drop of blood collects, dip the end of the bent limb of the capsule into it and allow the blood to pass into the capsule by capillary action. The capsule should, if possible, be filled three-quarters full. If blood does not flow freely, pressure may be applied by winding a narrow bandage from the base towards the finger-tip. A second puncture may facilitate the filling of the capsule. The success of the method depends on getting a *continuous* free flow of

blood. If there is any delay and the blood coagulates in the capillary part of the capsule before the required amount is collected, an additional capsule must be used. To close the capsule, heat the straight end in the flame and seal off the tip. As this end cools, the blood is retracted from the bent limb, which may then be sealed without heating or charring the blood. Both ends must be *completely* sealed.

When the blood coagulates in the capsule, the serum can be separated by centrifugalisation, the bent limb serving to hang the capsule on the rim of the centrifuge tube. A hand centrifuge is sufficient for this purpose. The capsule is opened by filing and breaking it at the bent end, and the serum is drawn off by means of a teat and capillary pipette.

THE AGGLUTINATION TEST

Apparatus required:

1 c.c. pipette graduated to the tip in 1/10ths and 1/100ths ; 0.1 c.c. pipette graduated to the tip in 1/100ths and 1/500ths ; a rubber teat, or preferably a mouth-piece for pipetting by suction—*i.e.* 3 in. of No. 5 quill tube with 9–12 in. of rubber tubing attached which can be connected with the top of the pipette (*vide infra*). The free end of the mouth-piece is “smoothed” in the Bunsen flame.

Steriliser with boiling water for pipettes, etc. (*vide* p. 48) ; sterile 0.85 per cent. saline ; test-tubes $3 \times \frac{1}{2}$ in. ; agglutination tubes $3 \times \frac{1}{8}$ in. ; test-tube racks suitable for the tubes ; small beaker or conical test-glass for saline solution ; grease pencil for marking tubes ; capillary pipette.

Prepare a suspension of *B. typhosus* by adding in fractions 5 c.c. of saline to a well-grown twenty-four-hours agar slope culture and emulsifying the growth with the aid of a platinum loop.¹ The emulsion is decanted and allowed to stand for half-an-hour until bacterial clumps and fragments of agar have sedimented.

¹ This emulsion can be standardised to a suitable opacity—*e.g.* tube 8, Brown's opacity standards (*vide* p. 147).

The following geometrical series of dilutions of the serum are prepared in the small ($3 \times \frac{1}{2}$ in.) test-tubes :—

①	②	③	④	⑤	⑥	⑦
1 in 15	1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	CONTROL— NO SERUM

—e.g. take 0·1 c.c. serum + 1·4 c.c. saline—i.e. 1 in 15—add to tubes ① and ② each 0·4 c.c. of this; then add to ② 0·4 c.c. saline—i.e. 1 in 30; from ② withdraw 0·4 c.c. into ③ and add to ③ 0·4 c.c. saline—i.e. 1 in 60—and so on till a 1 in 480 dilution is obtained in tube ⑥; from this withdraw and discard 0·4 c.c., leaving 0·4 c.c.; in tube ⑦ place 0·4 c.c. saline only.¹

0·4 c.c. of the bacillary suspension is added to each tube and the pipette is then sterilised. The dilutions of serum are now :

①	②	③	④	⑤	⑥	⑦
1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	1 in 960	CONTROL— SUSPENSION —NO SERUM.

The mixtures are transferred with a capillary pipette to agglutination tubes, starting with tube ⑦. Incubate for one and a half hours and then leave for half-an-hour at room temperature, when observations are made. Clumping or agglutination can easily be detected with the naked eye; the tubes should be examined in a strong light. The flocculi also tend to sediment, and the deposit is easily perceptible in the narrow tubes.

If less than 0·1 c.c. of serum is available, the test quantity of diluted serum and bacillary emulsion must be adjusted accordingly.

¹ In actual practice it is convenient to add the 0·4 c.c. saline first to each of the tubes, 2–7.

THE METHOD OF PIPETTING WITH A GRADUATED PIPETTE AND MOUTH-PIECE ¹

The glass mouth-piece is held between the teeth at the right corner of the mouth, and the top of the pipette is supported between the first and second fingers of the right hand so that the rubber tube immediately above the end of the pipette can be compressed between the thumb and the base of the first finger. The fluid is drawn up (*e.g.* from a tube) into the pipette by suction until the column extends just above the required graduation mark. The end of the mouth-piece is then closed by the tongue, and the column of fluid is depressed to the particular level by gentle pressure on the rubber tubing between the thumb and forefinger. *With the tongue still firmly applied to the mouth-piece* this exact volume of fluid can be transferred from the original tube and then expelled from the pipette into another tube.

This method, for which the necessary skill is soon acquired by practice, permits of *accurate* and *rapid* measurements of even small volumes.

Note.—The glass tube of the mouth-piece can be sterilised by flaming.

OTHER AGGLUTINATION TESTS

The agglutination technique described is also applicable to *diagnostic tests with B. paratyphosus A and B, B. melitensis, B. proteus* (Weil-Felix reaction of typhus fever), etc. The series of dilutions tested can, of course, be varied according to the lowest dilution in which agglutination would be accepted as a diagnostic criterion (*vide* pp. 209, 234, 288). It is essential in all cases to make these tests quantitative so that the highest dilution ("end-titre") in which agglutination results can be estimated.

¹ See also *Applied Bacteriology*, Browning, pp. 52–55.

AGGLUTINATION TESTS USED FOR THE SEROLOGICAL IDENTIFICATION OF CERTAIN ORGANISMS BY MEANS OF SPECIFIC ANTISERA

These tests are carried out by a similar technique. In this case the series of dilutions will depend on the "end-titre" of the serum for the homologous organism. Thus, if the end-titre were 1 in 16,000, the following range of dilutions might be tested:—1 in 1000 to 1 in 32,000 in geometrical series. For identification the unknown organism should agglutinate in approximately as high dilutions as a known homologous organism.

PREPARATION OF AGGLUTINATING ANTISERA

The instructions given here apply particularly to organisms of the typhoid-paratyphoid group.

Rabbits are used for immunisation, and large healthy animals should be selected, not under 2000 grams in weight.

The purity and identity of the culture used should be carefully ascertained beforehand.

The rabbits are injected intravenously (*vide* p. 125) at weekly intervals with a suspension in saline of a twenty-four-hours slope culture killed by exposure for one hour at 60° C. The following series of doses may be given:—1/20, 1/10, 1/5, 1/3 and 1/2 culture. These doses are easily measured by emulsifying a slope culture in a given volume of saline and then injecting the appropriate fraction.

In the case of organisms of high toxicity—*e.g.* *B. dysenteriae* Shiga—it is necessary to start with even lower doses—*e.g.* 1/100 of a culture.

Other methods for standardising doses may be used—*e.g.* where the doses are stated in terms of the number of organisms, as in the administration of vaccines (*vide* p. 146)—but the system indicated above is simple and sufficiently accurate for ordinary purposes.

Ten to twelve days after the last injection a specimen of blood is withdrawn from an ear vein (*vide* p. 126),

and the serum is tested for its agglutinating power with the strain used for immunisation. A series of dilutions are tested, and if agglutination occurs in a 1 in 1600 or higher dilution,¹ the animal is bled (*vide* p. 70), the blood is allowed to coagulate in a sterile measuring cylinder (plugged with cotton-wool), placed overnight in the ice-chest, and the serum is then separated. 0·1 c.c. of a 5 per cent. solution of phenol in salt solution is added for each c.c. of the serum—*i.e.* 0·5 per cent. pure phenol. This prevents bacterial growth resulting from any accidental contamination. The serum is stored in sterile quill tubes, about 1 c.c. in capacity, drawn out at both ends to capillary dimensions. The tubes are filled by suction, applying the mouth-piece used in pipetting (*vide* p. 134), and the ends are sealed in the Bunsen flame. The serum should be kept in the ice-chest, and may be expected to retain its potency for long periods (a year or more).

WASSERMANN SYPHILIS REACTION

This reaction depends on the “absorption” or “deviation” of haemolytic complement by an emulsion of certain lipoid substances in combination with the serum of a person infected with syphilis, and represents an important diagnostic test.

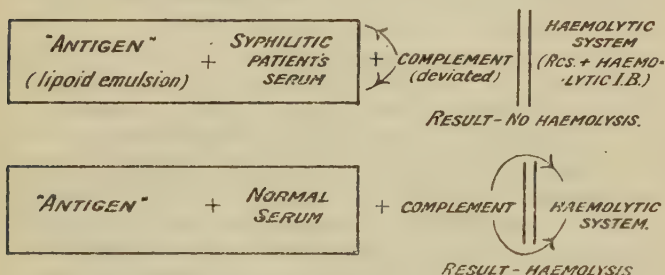
The effect is not a true immunity reaction, though the lipoid emulsion *plus* syphilitic serum deviates complement in the same way as a true bacterial antigen *plus* its homologous antiserum (*vide* p. 29).

For complement-deviation tests an indicator of the presence of complement is required. The “haemolytic system” used in these tests serves this purpose. It consists of the red corpuscles of a particular animal species “sensitised” with the corresponding haemolytic immune body (I.B.)—*e.g.* the red cells of the ox *plus* the serum of a rabbit which has been immunised

¹ More powerful agglutinating sera may of course be obtained; in immunising animals, 1 in 1600 is merely the *minimum* end-titre which should be aimed at.

with ox red cells. The immune body in the serum is thermostable. The serum is heated at 57° C. to remove natural complement and stored in tubes. The heated serum is non-haemolytic by itself, but in the presence of a suitable complement produces lysis of the homologous red corpuscles. Deviation of complement is denoted by the absence of lysis in the haemolytic system.

In its simplest form the Wassermann reaction can be represented as follows :—



REAGENTS

"ANTIGEN"

20 grams of sheep's heart-muscle, carefully freed from fat and fibrous tissue, are *finely* ground with sand in a mortar, and extracted for four days at room temperature with 100 c.c. 96 per cent. alcohol. The extract is filtered, and pure cholesterol is dissolved in it to the point of saturation.

For the test, a suspension is prepared by adding 1 part of the alcoholic extract to 12 parts of normal saline solution. In preparing the emulsion the *maximum turbidity* should be obtained by running the extract slowly on to the salt solution in a cylindrical measure (or test-tube), and then mixing slowly by rotation of the cylinder held at first in the vertical position.

PATIENT'S SERUM

A specimen of blood is obtained usually by vein puncture (*vide* p. 131) as for blood culture. The blood is allowed to coagulate in a sterile test-tube. It is advisable to obtain about 5 c.c. of blood. The serum is pipetted off after separation and heated at 55° C. for half-an-hour in a water-bath. Heating eliminates the fallacy of non-specific deviation effects that may occur with normal sera *plus* the antigen, and also deprives the serum of its own complementing property.

COMPLEMENT

The fresh serum of a guinea-pig is used. It contains an active haemolytic complement for the red corpuscles of the ox or sheep sensitised with the homologous haemolytic antibody. The blood is drawn, twelve to eighteen hours before the test, by severing the large vessels of the neck over a 6 in. funnel, from which the blood is collected in a measuring cylinder; it is allowed to coagulate and stand overnight in the ice-chest. The complement in serum too recently withdrawn is apt to be excessively deviable and, in consequence, is unsuitable for the Wassermann test.

Note.—Complement is unstable and deteriorates on keeping at ordinary temperatures. It is advisable throughout the experiment to keep the complement on ice.

HAEMOLYTIC SYSTEM

(Rcs + I.B.).—With guinea-pig's complement a haemolytic system consisting of ox or sheep red corpuscles, sensitised with the appropriate immune serum is used.¹

Defibrinated blood is obtained at the abattoir (*vide* p. 67) and washed three times with normal saline,

¹ We have found Burroughs Wellcome haemolytic serum for sheep corpuscles convenient and reliable.

being centrifugalised after each washing to separate the corpuscles. The washed cells are finally suspended in normal saline to form a 3 per cent. suspension, and five minimum haemolytic doses (M.H.D.) of the immune serum are added.

The method of preparing a HAEMOLYTIC ANTISERUM and of estimating its M.H.D. may be summarised as follows :—a rabbit is injected intravenously at weekly intervals with increasing amounts of washed red cells—*e.g.* 0·5 c.c., 1·0 c.c., 1·5 c.c. of the sediment after washing and centrifugalisation. A week after the last injection, a small quantity of blood is withdrawn from an ear vein; the serum is separated, and its M.H.D. estimated by testing the haemolytic effect of varying amounts (*e.g.* from 0·001 c.c. to 0·005 c.c.) on 1 c.c. 3 per cent. suspension of red cells along with an excess of guinea-pig complement (*e.g.* 0·05—0·075 c.c.) (*vide* methods given below). If the M.H.D. is 0·001 c.c.—0·002 c.c. the animal is bled from the neck vessels or by cardiac puncture (*vide* p. 70). The blood serum is separated, heated for one hour at 57° C., and stored in sealed quill tubes (*vide* p. 138).

THE TEST

Apparatus required:

Small test-tubes, $3 \times \frac{1}{2}$ in.; a rack for the tubes; graduated pipettes as used in the agglutination technique (*vide* p. 134).

As specimens of complement from different guinea-pigs vary quantitatively in their haemolytic effect, it is necessary first to estimate the M.H.D. (minimum haemolytic dose) of the complement to be used. The M.H.D. is the smallest amount required to produce complete haemolysis of the given quantity of red cells in the presence of excess of immune body. This usually lies between 0·004 c.c. and 0·01 c.c. of undiluted guinea-pig serum for 0·5 c.c. of the haemolytic system.

The following exemplifies the determination of the M.H.D. of complement :—

Tube . . .	1	2	3	4	5	6
Rcs + 5 M.H.D. of I.B. . . .	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.
Undiluted com- plement . . .	0·002 c.c.	0·004 c.c.	0·006 c.c.	0·008 c.c.	0·01 c.c.	—
Haemolysis .	Nil	Distinct	Marked	Just complete	Com- plete	Nil

The M.H.D. is therefore 0·008 c.c.

Note.—To measure amounts less than 0·01 c.c. make a 1 in 10 dilution of the serum, and of the diluted serum take ten times the actual amount required, using the 0·1 c.c. pipette graduated in 1/100ths and 1/500ths.

The actual test is shown in the following Table :—

	<i>Test</i>			<i>Serum Controls</i>		<i>Antigen Controls</i>	
Tube .	1	2	3	1	2	1	2
Normal saline .	—	—	—	0·5 c.c.	0·5 c.c.	—	—
Antigen .	0·5 c.c.	0·5 c.c.	0·5 c.c.	—	—	0·5 c.c.	0·5 c.c.
Patient's serum .	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.	—	—
Comple- ment .	4 MHD	8 MHD	14 MHD	2 MHD	4 MHD	2 MHD	4 MHD

Complement dose Control :—

Tube . . .	1	2	3	4
Saline . . .	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.
Complement .	0·004 c.c.	0·006 c.c.	0·008 c.c.	0·01 c.c.

The mixtures are incubated for one and a half hours, and then to each tube 0·5 c.c. of the haemolytic system is added. The tubes are again incubated for one hour, when the results are noted. Absence of haemolysis in the test series signifies that

deviation of complement has occurred, so that not even 1 M.H.D. is left free. The serum and antigen controls determine any possible anti-complementary action of either of these reagents, and the complement dose control indicates any possible deterioration in the haemolytic value of the complement by dilution and incubation. By carrying out the test in this quantitative way the number of doses of complement deviated by antigen *plus* serum can be estimated—*i.e.* the strength of the reaction. Known negative and positive sera must always be included in any set of Wassermann tests as controls.

The practical details and interpretation of the results can only be learned by actual acquaintance with the test.

In this method, 4 M.H.D. is the smallest amount of complement *generally* used for the actual test. The inclusion of still smaller amounts (*e.g.* 2 M.H.D. or 3 M.H.D.) introduces the fallacy of non-specific deviation with normal sera.

It must be remembered that specimens of complement from different guinea-pigs vary in deviability. With a weakly-deviable complement it may even be impossible to elicit positive reactions, while with a strongly-deviable complement non-specific effects are rendered more marked. The controls with known negative and positive sera are therefore indispensable.

Absence of lysis even in tube 1 is to be regarded as a positive result, provided lysis occurs with 2 M.H.D. of complement in the serum and antigen controls and provided the known negative serum shows complete lysis in tube 1. Strongly reacting sera may show absence of lysis in tubes 1, 2 and 3. With a negative serum complete lysis usually occurs in tube 1. Any anti-complementary effect of the serum or antigen must be allowed for in estimating the deviation by serum + antigen, and the results with known positive and negative sera are to be regarded as standards for comparison with the results given by the sera tested.

**EXAMPLES OF THE RESULTS OF WASSERMANN TESTS
CARRIED OUT BY THE METHOD DESCRIBED ABOVE**

<i>Tube</i>	1	2	3	<i>Serum Control</i>	<i>Antigen Control</i>
Amount of comple- ment in M. H. D.	4	8	14	2	2
Amount of comple- ment in c.c.	0·024	0·048	0·084	0·012	0·012
Negative serum	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis
Positive serum	No lysis	Trace of lysis	Complete lysis	Complete lysis	—
“Weak positive” serum	No lysis	Almost complete lysis	Complete lysis	Complete lysis	—
“Marked positive” serum	No lysis	No lysis	No lysis	Complete lysis	—

Complement dose :—

0·004
Marked
lysis

0·006
Just complete
lysis

0·008
Complete
lysis

(In this experiment the M.H.D. of complement is 0·006 c.c. both in the initial titration and in the complement dose control.)

**THE WASSERMANN REACTION WITH CEREBRO-
SPINAL FLUID**

In testing spinal fluid the reaction is carried out as in the case of serum, but with the following modifications :—

- (1) The antigen is prepared by emulsifying 1 part of the cholesterolised alcoholic extract with 12 parts of spinal fluid ; 0·5 c.c. of this mixture replaces antigen + serum in the usual test.

- (2) In place of saline + serum in the usual serum controls, 0.5 c.c. of spinal fluid is tested.
- (3) The cerebro-spinal fluid is not heated to 55° C. before the test as in the case of serum.

THE SACHS-GEORGI REACTION

This reaction is a serological diagnostic test for syphilis, and depends on the occurrence of flocculation in mixtures of syphilitic serum and the lipoid antigen used in the Wassermann test. The results are more or less parallel to those of the complement-deviation test, and the flocculation reaction may be substituted for it.

The reaction is carried out in a manner similar to an agglutination test (*vide* p. 135).

The patient's serum is heated at 55° C. for half-an-hour, and a series of dilutions in saline are prepared in small test-tubes as follows :—

①	②	③	④	⑤	⑥
<i>1 in 2</i>	<i>1 in 4</i>	<i>1 in 8</i>	<i>1 in 16</i>	<i>1 in 32</i>	<i>1 in 64</i>

0.4 c.c. of each dilution is a convenient quantity for the test, and to each tube one-half of this volume (0.2 c.c.) of the Wassermann antigen, made up as a 1 in 6 suspension by slow admixture with normal saline, is added. The mixtures are then transferred to narrow agglutination tubes, incubated at 37.5° C. for four hours, and allowed to stand overnight at room temperature, when readings are made of the results. A control tube should be included containing antigen emulsion and saline, but no serum. With strongly reacting sera, flocculation may occur even in the highest dilution of the series. With weak sera definite flocculation may occur only in tubes 1 and 2. "Zone phenomena" are sometimes noticed—*i.e.* where flocculation is less with lower dilutions of serum than with higher dilutions.

Note.—The reaction can be hastened by shaking the mixtures of serum and antigen for five to ten minutes.

This can easily be done after the addition of the antigen to the serum dilutions, by placing the small test-tubes vertically in a shaking machine (such as that used for shaking a Winchester quart bottle). The tubes are held in racks fixed to the carrier of the machine.

PREPARATION OF BACTERIAL VACCINES

The particular organism (or organisms) must be isolated in pure culture, and then several cultures are made on appropriate solid medium so as to yield after twenty-four to forty-eight hours incubation sufficient growth, according to the amount of vaccine to be prepared, and the abundance of the growth on the particular medium. The growth is emulsified in sterile carbol-saline (0.85 per cent. sodium chloride + 0.5 per cent. carbolic acid) so as to form a fairly dense emulsion. The emulsion should be free from fragments of medium. If present they can be removed by centrifugalising the emulsion for two or three minutes, or by allowing them to sediment by gravity, and then decanting the supernatant fluid. The bacterial suspension must be rendered as uniform as possible by shaking in a tube or bottle with glass beads. A special shaking machine is generally used for this purpose. *All manipulations involved in preparing the suspension must be carried out with strict precautions to avoid contamination.*

STANDARDISATION

It is necessary at this stage to estimate the *approximate* number of bacteria per c.c. of the suspension. Various methods are available for this purpose. Those recommended are :

1. *Haemocytometer Method.*—Use a Thoma cell ; make a 1 in 20 dilution of the suspension—*e.g.* 0.1 c.c. suspension + 1.8 c.c. saline + 0.1 c.c. centrifugalised Loeffler's methylene blue—and add two loopfuls of pure formalin to kill the organisms to be counted ; mount a drop on the haemocytometer stage as in blood-counting and count the number of organisms in 20–50

small squares in different parts of the cell; find the average number per square and multiply this by 80,000,000; the result is the approximate number of bacteria per c.c. in the original suspension.

2. *Comparison with Standard Opacity Tubes (Brown).*¹
—This consists in comparing the opacity of the suspension with that of a series of ten standard tubes containing different dilutions of barium sulphate suspended in 1 per cent. sodium citrate. In making comparisons the bacterial suspension should of course be placed in a similar tube to the standards. The standard tubes must be thoroughly shaken. The matching is facilitated by reading printed letters through the suspensions.

The following table gives the numerical equivalents of the opacity standards for certain organisms according to Cunningham and Timothy²:—

SHOWING THE RELATION OF OPACITY TO THE NUMERICAL EQUIVALENT OF VARIOUS BACTERIA ESTIMATED BY MEANS OF THE HAEMOCYTOMETER METHOD

Opacity Tube No.	<i>Staphylococcus aureus</i>	<i>Streptococcus, haemolytic</i>	<i>Pneumococcus</i>	<i>Gonococcus</i>	<i>B. coli</i>	<i>B. typhosus</i>	<i>B. para-typhosus B</i>	<i>B. dysenteriae (Flemer)</i>	<i>B. influenzae (Pfeiffer)</i>
10	3789	3043	7053	3578	3787	4577	4171	4494	11,396
9	3410	2739	6348	3220	3408	4119	3754	4045	10,256
8	3031	2434	5642	2862	3030	3662	3337	3595	9117
7	2652	2130	4937	2505	2651	3204	2920	3146	7977
6	2273	1826	4232	2147	2272	2746	2503	2696	6838
5	1895	1522	3527	1789	1894	2289	2086	2247	5698
4	1516	1217	2821	1431	1515	1831	1668	1798	4558
3	1137	913	2116	1073	1136	1373	1251	1348	3419
2	758	609	1411	716	757	915	834	899	2279
1	379	304	705	358	379	458	417	449	1140

The figures represent millions per c.c.

¹ See Brown, H. C., *Indian Journal of Medical Research*, 1919, vii., No. 1, pp. 238-250.

² See *Indian Journal of Medical Research*, 1924, xi., No. 4, p. 1253.

3. *Gates's Method*.¹—The opacity of a bacterial suspension is measured by the length of column of the suspension required to cause the disappearance of a wire loop. By means of a simple formula the approximate number of bacteria per c.c. can readily be determined. Gates has devised an instrument for the purpose which is easily made: the details are given in his article. We have found this method of estimating the number of organisms in a suspension to be simple, rapid and reliable.

STERILISATION OF THE VACCINE

The suspension is sterilised at relatively low temperatures—*e.g.* 60° C. for one hour in a vaccine bath. The sterility is then tested by transferring six loopfuls to a tube of suitable medium and incubating for forty-eight hours.

PREPARATION OF THE VACCINE FOR ADMINISTRATION

Any series of graduated doses consisting of a certain number of organisms (usually computed in millions) can be prepared in volumes of 1 c.c. by making appropriate dilutions in carbol-saline from the original standardised suspension. Graduated pipettes, as used in serological work, are employed for the purpose. The dilutions are made in sterile tubes and each dose is placed in a sterile vaccine ampoule.

The most convenient method of supplying the vaccine for actual use is to prepare from the stock suspension, concentrations of 50, 100, 500 or 1000 million organisms per c.c. (according to the doses required) in quantities of 20 c.c. The dilutions are placed in 25 c.c. "vaccine bottles" with special tightly fitting thick rubber caps which are covered with a layer of paraffin wax. The required dose can be obtained by puncturing the cap with the hypodermic syringe and withdrawing the appropriate amount.

¹ Gates, F. L., *Journal of Experimental Medicine*, 1920, xxxi., pp. 105-114.

In preparing dilutions from the stock vaccine all manipulations, etc., must be carried out with strict precautions to prevent contamination. Pipettes, tubes, ampoules, bottles, caps, etc., must be absolutely sterile.

Before supplying the diluted vaccine it is advisable to carry out further sterility tests, using the contents of one of the ampoules, or 0.5 c.c. withdrawn from the bottled vaccine with a syringe.

When a vaccine representing more than one type of organism is required—*e.g.* for mixed infections—pure cultures of each organism must be obtained and separate standardised suspensions prepared. Appropriate concentrations of each are then combined in the final preparation.

BACTERIOLOGICAL EXAMINATION OF WATER

From the hygienic standpoint, the bacteriological examination of water resolves itself into the determination of the presence or absence of any serious excretal contamination.

Under certain conditions, *B. coli* represents the most reliable indicator of such pollution.

As this group of organisms is derived from the intestine of various animals, even water supplies far removed from human contamination may contain it in small numbers. In water grossly polluted with excretal matter (*e.g.* sewage), *B. coli* is present in large numbers. If it is to be accepted as an indicator of purity, the test for its presence must be carried out on a quantitative basis, so that the *B. coli* content of the water can be definitely estimated.

This group of bacteria includes a large number of different types. Some of these ("typical") are more prevalent in faeces than others ("atypical") and obviously the former are of more significance as indicators of recent faecal contamination. In carrying out the test for *B. coli* in water, it is essential to determine whether the types present are "typical" or "atypical" (*vide* pp. 199, 200).

The presence of streptococci and sporing anaerobic bacilli is of additional significance in determining water purity. The occurrence of streptococci is of course proof positive of recent faecal pollution, but their absence does not exclude such impurity. The intestinal sporing anaerobes, being highly resistant, do not by themselves necessarily indicate a recent or serious pollution.

The enumeration of the total viable bacteria in a water specimen is also a useful supplementary test in determining its purity, but this test is of little value by itself.

The demonstration of pathogenic bacteria (*e.g.* *B. typhosus*) would obviously constitute the most direct proof of a serious impurity, but pathogens, if present, are usually so scanty that the technical difficulty of their isolation makes this test impracticable for ordinary purposes.

The routine tests used in the bacteriological examination of water are:

- (1) Enumeration of the viable bacteria.
- (2) The quantitative *B. coli* test.

Collection of specimens.—Specimens are taken in 6 oz. stoppered bottles sterilised by dry heat. When drawn from a tap, the mouth of the tap should be flamed if possible, and the water allowed to run for ten minutes before filling the bottle. In the case of streams, rivers and lakes, the bottle is filled by dipping the mouth (with the stopper in position) below the surface, and then opening the bottle under water by removing the stopper with forceps. This avoids the collection of surface water, which may contain a good deal of decomposing vegetable matter. When a sample is to be obtained from a depth, a bottle weighted with lead is used, having two cords attached—one to the neck, the other to the stopper; the bottle is lowered to the required depth, and is filled by jerking out the stopper by means of the attached cord; the bottle is then quickly raised to the surface and re-stoppered.

When a certain length of time must elapse before

the laboratory examination can be carried out, the bottles should be kept on ice. Special ice-boxes for the purpose can be obtained, and are essential where specimens have to be transported some distance.

Enumeration of viable bacteria.—With a sterile pipette add 2 c.c. water to 10 c.c. nutrient agar (standardised to + 1, Eyre's scale), melted and cooled to 50° C.; mix thoroughly, pour in a Petri dish, and allow to solidify.

If the water is suspected of contamination, add a smaller quantity—*e.g.* 0.1 c.c.—and in dealing with specimens of uncertain purity it is advisable to make a series of plate cultures with varying quantities of the water. The agar should be as transparent as possible.

Make duplicate plates in this way and incubate one at 37.5° C., the other at 22° C., for three days.¹

Count the colonies that develop in the medium. To aid counting, divide the plate into sections by ruling on the glass with a grease pencil. Count the colonies in each section and add the numbers. Each colony may be taken to represent one viable bacterium in the original specimen.

The number of viable bacteria in the average municipal water supply should not usually exceed 100 per c.c.

In uncontaminated water, the number of colonies developing at 22° C. is usually greater than at 37.5° C., as the organisms present are mainly saprophytes.

B. Coli Test—

Media required:

- (1) 2 per cent. peptone water containing 0.5 per cent. sodium taurocholate, 1 per cent. lactose, and neutral red as indicator (*vide* p. 65).
- (2) Same constituents as (1), but in double concentration.

Fixed amounts of these media are put up in 50 c.c. sterile cylindrical bottles stoppered with cotton-

¹ Nutrient gelatin is often used for determining the number of organisms viable at 22° C.

wool plugs, and containing a small inverted tube for indicating gas production. The amounts are as follows :—

5 c.c. of the single strength medium	
5 c.c. of the double strength	„
10 c.c.	„ „
20 c.c.	„ „

The medium, after bottling, is sterilised at 100° C. for twenty minutes in the Koch's steriliser on three successive days. With sterile pipettes the following amounts of water are added¹ :—

0.1 c.c. to	5 c.c. of the single strength medium
0.5 c.c. „	5 c.c. „ „
1 c.c. „	5 c.c. „ „
5 c.c. „	5 c.c. of the double strength medium
10 c.c. „	10 c.c. „ „
20 c.c. „	20 c.c. „ „

The bottles are incubated for forty-eight hours, and then sub-inoculations are made on MacConkey plates from those which show acid, or acid and gas production. The plates are incubated for twenty-four hours, and red colonies are picked-off on to agar slopes, and the resulting pure growths are put through the following tests to ascertain whether they represent "typical" *B. coli* :—

Fermentation of	{ <i>Lactose</i> <i>Adonite</i> <i>Inosite</i>
Production of	<i>Indol</i>
Liquefaction of	<i>Gelatin</i>
(vide pp. 199, 200)	

The fermentation of lactose may be assumed from the original test, but it should be confirmed by a subsequent test with the pure culture isolated.

¹ This should be done at the place where the water specimen is taken—*i.e.* on the spot—especially if it is some distance from the laboratory.

A typical *B. coli* shows the following reactions :—

Lactose	+
Adonite	—
Inosite	—
Indol	+
Gelatin	—

The smallest amount of water which contains typical *B. coli* can then be stated.

A pure water should not contain typical *B. coli* in 10 c.c. or less, and in the case of a specimen from a suspicious source with typical *B. coli* in 10 c.c., the water can justifiably be condemned for drinking purposes.

BACTERIOLOGICAL EXAMINATION OF SEWAGE AND SEWAGE EFFLUENTS

The bacteriological examination of sewage is of relatively little practical importance, unless in determining the condition of an effluent which is being discharged into a stream from which drinking water is obtained, or in determining the purity of an effluent from a sewage purification process.

The procedure is the same as in water examination ; an estimation of the viable bacteria present is made by plating, and counting colonies, and the *B. coli* test is carried out as with a specimen of water ; much smaller amounts, however, are tested than in the case of water, depending on the likely extent of dilution of the effluent.

In routine work the following amounts should be tested for *B. coli* by the method described under water examination (*vide supra*):—

- | | | |
|-----|--------------------------------|--|
| (1) | 0·1 c.c. of effluent undiluted | |
| (2) | „ „ diluted | 1 in 10— <i>i.e.</i> 0·1 c.c. undiluted effluent
+ 0·9 c.c. sterile water |
| (3) | „ „ „ | 1 in 100— <i>i.e.</i> 0·1 c.c. effluent 1 in 10
+ 0·9 c.c. sterile water |
| (4) | „ „ „ | 1 in 1000 — and so on in series |
| (5) | „ „ „ | 1 in 10,000 |
| (6) | „ „ „ | 1 in 100,000 |
| (7) | „ „ „ | 1 in 1,000,000 |

It is generally unnecessary to determine whether the lactose-fermenters present are typical or atypical *B. coli*.

If an estimation of the total viable bacteria is required, quantities of 0.5 c.c. of dilutions (3), (5) and (7) are plated as in water examination (*q.v.*). The particular plates in the series most suitable for colony counts will of course depend on the bacterial content of the specimen. In estimating numbers of bacteria per c.c., allowance must be made for the particular dilution. The numbers per c.c. in crude sewage vary greatly—*e.g.* from 1–100 million.

BACTERIOLOGICAL EXAMINATION OF MILK

In hygiene work the bacteriological examination of milk generally consists in :

- (1) An estimation of the number of bacteria present in a given quantity.
- (2) An estimation of the degree of contamination present from faecal sources—*e.g.* a quantitative *B. coli* test.
- (3) The determination of the presence of specific pathogens—*e.g.* *B. tuberculosis*.

The specimen of milk is collected in an 8 oz. sterile stoppered bottle, and, if there should be any unavoidable delay in carrying out the tests after collection of the sample, the bottle must be kept on ice ; if it has to be transported some distance, a special ice-box should be used similar to that usually employed for the transmission of water specimens.

Dilutions of 1 in 10, 1 in 100, 1 in 1000, and 1 in 10,000 (with sterile water as diluent) are prepared in sterile stoppered bottles, by adding 10 c.c. of the undiluted milk to 90 c.c. water—*i.e.* 1 in 10, 10 c.c. of the 1 in 10 dilution to 90 c.c. water—*i.e.* 1 in 100, and so on. The dilutions are thoroughly mixed by shaking.

1. The number of bacteria per c.c. capable of growing in a nutrient agar standardised + 1 by Eyre's

method is estimated by plating 0.5 c.c. from each of the decimal dilutions (as in water examination), incubating for forty-eight hours at 37.5° C., counting the colonies in one of the plates that is not overcrowded, and calculating from this the number of viable organisms per c.c. If the milk is grossly contaminated, platings from even higher dilutions may be necessary in order to obtain a plate in which the colonies are not too numerous for counting purposes.

Note.—Under the most favourable conditions a specimen of milk will contain at least 500 bacteria per c.c.; but under bad conditions the numbers may reach even millions per c.c. Milk that has been produced with special precautions to avoid undue contamination is recognised for consumption as “certified milk.” Thus, in Scotland, “Certified Milk” must not contain more than 30,000 bacteria per c.c. when delivered to the consumer or at any time prior to delivery.

2. Varying amounts of milk are added to tubes or bottles of lactose-bile-salt medium (as in the *B. coli* water test, p. 151). The range of amounts that require to be tested depends on the likely degree of contamination. In the case of ordinary dairy milk the following series is suggested:—

1.0 c.c. }	Undiluted milk	
0.1 c.c. }		
0.1 c.c. of a	1 in 10	dilution of the milk
“ “	1 in 100	“ “
“ “	1 in 1000	“ “
“ “	1 in 10,000	“ “
“ “	1 in 100,000	“ “

In some cases even smaller amounts may have to be tested. The decimal dilutions are prepared in series (*vide supra*).

The smallest amount which yields acid *and* gas production (due to *B. coli*) is ascertained.

Vended milk should not contain more than 100–500 *B. coli* per c.c. in winter, and 1000–2500 in summer (Savage).

3. *Examination for tubercle bacilli*.—100 c.c. of milk, divided into 25 c.c. amounts, are centrifugalised for half-an-hour at a minimum speed of 3000 revolutions per minute. The sediments, after pipetting off all but 1 c.c. of the milk in each centrifuge tube, are mixed and examined microscopically for tubercle bacilli (*vide* p. 102). Before staining, the dried films may be treated with ether for some hours to remove the fat. At the same time several guinea-pigs are injected subcutaneously with the sediment and kept under observation to ascertain whether tuberculous lesions result (*vide* p. 185). It is necessary to inoculate a number of animals from one specimen, as a certain proportion of the inoculated guinea-pigs may die from infection with other organisms present in the milk—*e.g.* sporing anaerobic bacilli. This difficulty may be obviated to some extent by using fresh specimens.

The microscopic examination may reveal acid-fast bacilli other than the tubercle bacillus (*vide* p. 189). The absence of tubercle bacilli in films does not exclude their presence in the specimen. Hence the inoculation test should be relied on rather than the direct method.

Other pathogens in milk.—The methods for demonstrating *B. typhosus* and *B. diphtheriae* correspond to those used for the isolation of these organisms. For *B. typhosus*, the sediment, after centrifugalisation, should be plated out on the surface of MacConkey's medium (*vide* p. 205). For *B. diphtheriae*, Loeffler's serum slopes are inoculated with the sediment (*vide* p. 181).

TESTING OF GERMICIDES

By "germicide" is meant a substance which destroys micro-organisms. The terms "bactericide," "antiseptic" and "disinfectant" are usually employed as synonyms, though "antiseptic" is more accurately applicable to substances which inhibit bacterial growth. Most substances, however, which

inhibit growth are germicides if used in sufficiently high concentration, or if the exposure is sufficiently long.

THE "INHIBITION COEFFICIENT" is the lowest percentage concentration of a particular antiseptic or germicide that will completely inhibit growth in nutrient medium—*e.g.* 1 in 400 carbolic acid for *B. typhosus*.

One of the best methods of determining this is to incorporate a series of different concentrations of the antiseptic in 10 c.c. nutrient agar, pour the medium in plates, and then make a stroke inoculation from a bacterial emulsion. The emulsion should be prepared in sterile saline from a young culture on solid medium, and should show just a faint turbidity to the naked eye. Different organisms can be tested at the same time by making stroke inoculations from each on the various plates. The surface of the medium must be free from condensation fluid.

The plates are incubated for forty-eight hours, when observations can be made.

Instead of solid medium, tubes of bouillon may be used.

THE "INFERIOR LETHAL COEFFICIENT" expresses the concentration of a germicide and the time of exposure required to kill non-sporing bacteria—*e.g.* 1 in 60 carbolic acid kills streptococci in five minutes.

It can be determined by preparing a series of concentrations of the substance in a fixed quantity of diluent, adding a constant quantity of bacterial emulsion, and transferring at intervals a certain amount of the mixture to some suitable culture medium (*e.g.* bouillon; or melted agar at a temperature of 50°C., which, after the transfer, is poured in plates).

The quantity of antiseptic carried over to the culture medium must not be equal to or above the inhibition coefficient.

THE "SUPERIOR LETHAL COEFFICIENT" expresses the concentration of a germicide and the time of exposure required to kill bacterial spores—*e.g.* 1 in 2000

perchloride of mercury kills anthrax spores in twenty-six hours.

The **PHENOL COEFFICIENT** expresses the germicidal power of a particular substance as compared with pure phenol.

Method of Testing—

- (1) Determine beforehand the inhibition coefficient of the particular germicide for *B. typhosus* and make up a series of four graded concentrations, starting with a concentration slightly greater than the inhibition coefficient.
- (2) Make up the following phenol solutions :—
1 per cent. 0·9 per cent. 0·8 per cent. 0·7 per cent.
- (3) Emulsify a twenty-four-hours agar slope culture of *B. typhosus* in 10 c.c. sterile water. Shake thoroughly in a sterile bottle containing glass beads and centrifuge in a sterile tube for two minutes. The supernatant emulsion when pipetted off constitutes a uniform suspension.
- (4) To 5 c.c. of each of the solutions prepared from (1) the germicide to be tested, and (2) the phenol, add with a sterile pipette 0·1 c.c. of the bacterial emulsion and shake the mixtures. The tubes containing the solutions should be kept during the test in a water-bath at 18° C.
- (5) At intervals of $2\frac{1}{2}$ minutes up to 15 minutes remove 0·1 c.c. with a sterile pipette from the mixtures and transfer to tubes of 10 c.c. bouillon.
- (6) Incubate the bouillon tubes for seven days, and note in which growth has occurred.

Note.—The greatest care must be taken in making the transfers to avoid contamination which may produce growths of other organisms in the bouillon tubes. If there is any doubt as to whether the resulting growth represents *B. typhosus* or some contaminant, cultural controls must be carried out.

(7) The coefficient is calculated as follows:—

Divide the figure representing the percentage strength of the weakest lethal dilution in the phenol test at $2\frac{1}{2}$ minutes exposure *by* the figure representing the percentage strength of the weakest lethal dilution of the unknown germicide also at $2\frac{1}{2}$ minutes exposure. The quotient is the coefficient at $2\frac{1}{2}$ minutes.

The corresponding coefficients are calculated for 5, $7\frac{1}{2}$, 10, $12\frac{1}{2}$ and 15 minutes exposure, and the mean of these is the final phenol coefficient.

The following results illustrate the test:—

			Time in Minutes					
			$2\frac{1}{2}$	5	$7\frac{1}{2}$	10	$12\frac{1}{2}$	15
Unknown Germicide	0.5 per cent.	.	—	—	—	—	—	—
	0.4	„	+	+	+	—	—	—
	0.3	„	+	+	+	+	+	—
	0.2	„	+	+	+	+	+	+
Phenol	1.0	„	—	—	—	—	—	—
	0.9	„	+	—	—	—	—	—
	0.8	„	+	+	+	—	—	—
	0.7	„	+	+	+	+	+	+

(+ = growth, — = no growth)

$$\text{Coefficient (} 2\frac{1}{2} \text{ minutes) } = \frac{1.0}{0.5} = 2.0$$

$$\text{„ (5 minutes) } = \frac{0.9}{0.5} = 1.8$$

$$\text{„ (} 7\frac{1}{2} \text{ minutes) } = \frac{0.9}{0.5} = 1.8$$

$$\text{„ (10 minutes) } = \frac{0.8}{0.4} = 2.0$$

$$\text{„ (} 12\frac{1}{2} \text{ minutes) } = \frac{0.8}{0.4} = 2.0$$

$$\text{„ (15 minutes) } = \frac{0.8}{0.3} = 2.66$$

$$\text{Mean coefficient} = 2.04$$

VIII

BACTERIOLOGICAL DIAGNOSIS

THE CHARACTERISATION AND OCCURRENCE
OF MICRO-ORGANISMS OF IMPORTANCE IN
MEDICINE, WITH THE METHODS OF BACT-
ERIOLOGICAL DIAGNOSIS IN THE VARIOUS
INFECTIONS

PYOGENIC COCCI

THESE organisms are found in acute inflammatory and suppurative conditions. They are associated with pus formation, and are therefore designated "pyogenic." The most frequent are :

Staphylococcus pyogenes aureus, and
Streptococcus pyogenes.

STAPHYLOCOCCUS AUREUS

Morphology.—Spherical cocci arranged in irregular clusters, the individual cells being approximately 0.8μ in diameter.

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe; temperature range— 10° – 42° C., optimum— 35° C.; growth occurs on ordinary nutrient media.

Agar stroke—thick, opaque, moist, shiny, "oil-paint"-like growth which develops a characteristic golden or orange-yellow colour.

Colonies on agar—circular discs, relatively large (2–3 mm. in diameter), and presenting the same characters as the stroke culture.

Gelatin—growth in this medium is associated with liquefaction due to a "gelatinase" ferment.

Bouillon—uniform turbidity, with subsequent orange-coloured deposit.

Milk—acid formation; and later coagulation.

Haemolysin is produced in culture.

Occurrence.—In localised abscesses, wound suppuration, skin pustules, furuncles and carbuncles, blepharitis, mucous catarrhs, acute osteitis, septicaemia, pyaemia, urinary sepsis, etc.

OTHER TYPES OF STAPHYLOCOCCI

Staphylococcus pyogenes albus.—Growth is similar to that of *Staphylococcus aureus*, but white and devoid of pigment.

Staphylococcus pyogenes citreus.—An uncommon type; differentiated from others by lemon-yellow colour of growth.

Staphylococcus cereus albus and *Staphylococcus cereus flavus.*—Uncommon types; develop wax-like growths, the former white, the latter yellow.

Note.—The staphylococci are also common skin, mouth and throat commensals, especially the *albus* type. The *aureus* type is the most virulent; the others are of relatively low virulence.

STREPTOCOCCUS PYOGENES

Morphology.—Spherical or oval cocci, $0.7-1\mu$ in diameter, in chains of variable length; involution forms frequent.

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe; temperature range—generally $20^{\circ}-42^{\circ}\text{C.}$, optimum— 37.5°C. ; grows on ordinary media, but better on blood or serum media.

Agar stroke—growth consists of small circular discrete semi-transparent discs, about 1 mm. in diameter after twenty-four hours incubation.

On blood agar—clear zone develops round the colonies, due to diffusible haemolysin produced by the organism.

Bouillon—growth forms as a granular sediment in the culture tube.

Biochemical Reactions.—Tested by growing in serum water medium with 1 per cent. of fermentable carbohydrate, and an indicator of acidity (litmus or neutral red):

Glucose	Lactose	Saccharose	Mannite	Raffinose	Inulin
⊥	⊥	⊥	—	—	—

(⊥ = Acid ; no gas)

OTHER TYPES OF STREPTOCOCCI OF MEDICAL IMPORTANCE

The streptococci constitute a somewhat heterogeneous group, and include types which are normal inhabitants of the mouth, throat and bowel.

In medical work these organisms have been broadly subdivided according to the appearances of growths on blood agar. Thus in the case of the *Strept. pyogenes*, zones of haemolysis develop round the colonies (*vide supra*), and this type has been classified as "*Strept. haemolyticus*." Certain varieties occurring as commensals in the mouth and throat—*e.g.* "*Strept. salivarius*"—are non-haemolytic, and their colonies on blood agar show a greenish coloration, due to met-haemoglobin production. These are often designated "*Strept. viridans*." In a general way, the pathogenic types can be differentiated from the non-pathogenic varieties by their haemolytic properties. It must be noted, however, that the "viridans" types are potential pathogens, and no exact parallelism exists between haemolysin production and pathogenicity.

Fermentative reactions have been extensively utilised for the differentiation of streptococci, but these reactions can hardly be regarded as sufficiently "fixed" characters for accurate classification. Certain biochemical tests, however, are of undoubted importance in the general grouping of the streptococci. Thus the "*Strept. faecalis*," the common type of streptococcus in human faeces, can be generally differentiated

from the "pyogenes" and "salivarius" types by its fermentation of mannite. It is also non-haemolytic. The "salivarius" type is characterised by the fermentation of raffinose, whereas *Strept. pyogenes* does not, as a rule, ferment either mannite or raffinose.

Thus, the three main types met with in medical bacteriology can be classified as follows (Gordon):—

	<i>Haemolysis</i>	<i>Raffinose Fermentation</i>	<i>Mannite Fermentation</i>
<i>S. pyogenes</i> . .	+	—	—
<i>S. salivarius</i> . .	—	⊥	—
<i>S. faecalis</i> . .	—	—	⊥

Certain other types have been recognised and named as follows:—

Strept. mucosus is capsulated and produces slimy colonies; it has been noted in various suppurative conditions.

"*Strept. anginosus*" is the designation that has been given to a pathogenic type associated with acute inflammatory conditions of the throat—*e.g.* in scarlatina. It is haemolytic and closely related to *Strept. pyogenes*.

The so-called "*Strept. rheumaticus*" is a non-haemolytic type which has been noted by some observers in the lesions of acute rheumatism (*e.g.* endocarditis). It is closely related biologically to the *Strept. faecalis*.

THE PATHOGENICITY AND OCCURRENCE OF STREPTOCOCCI

The pathogenic effects of the streptococci depend on the virulence of the particular strain and the susceptibility of the host. Thus a strain of low virulence may produce only a localised inflammation, whereas strains of high virulence may be associated with spreading inflammation and even septicaemia.

Haemolytic streptococci are found in the following conditions:—

Wound suppuration; localised abscesses; spreading inflammations—*e.g.* erysipelas, cellulitis, lymphangitis, lymphadenitis; septicaemia (*e.g.* puerperal septicaemia) and pyaemia; acute osteitis; suppurative otitis and mastoiditis; meningitis, usually secondary to some other lesion—*e.g.* otitis; ulcerative endocarditis; inflammation of the fauces, tonsils, pharynx and larynx—*e.g.* acute follicular tonsillitis, the angina of scarlet fever; broncho-pneumonia secondary to influenza, measles, etc.; peritonitis; cholecystitis; urinary sepsis, etc.

In septic lesions, mixed staphylococcal and streptococcal infections are frequent.

Streptococci may also occur as secondary invaders—*e.g.* in diphtheria, gonorrhoea, etc.

Streptococcus viridans types are usually non-pathogenic, but may occur as pathogens in lesions of a less acute form than those of the haemolytic varieties—*e.g.* cases of tonsillitis, otitis media, dental abscesses, broncho-pneumonia, subacute endocarditis. Even general infections of a septicaemic type may be due to these organisms.

Non-haemolytic streptococci have also been regarded as the causative organisms of acute rheumatism and its complications (*v.* *Strept. rheumaticus*), but the evidence is inconclusive.

MICROCOCCUS TETRAGENUS

Morphology.—Spherical cocci in tetrads, about 0.7μ in diameter, and sometimes capsulated.

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe; optimum temperature— 37.5°C. ; grows well on ordinary media.

Agar—growth resembles *Staph. albus*.

Gelatin—not liquefied.

Occurrence.—Suppuration in region of mouth and neck—*e.g.* dental abscess; cervical adenitis; pulmonary abscess, etc.

OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS

BACILLUS PYOCYANEUS

Morphology.—Straight rods; 1.5 to 3μ by 0.5μ ; motile with a terminal flagellum; non-sporing.

Staining.—Gram-negative.

Culture.—Aerobe and facultative anaerobe; temperature range— 18° – 43° C., optimum— 37.5° C.; grows on ordinary media.

Agar—stroke inoculation produces an abundant, moist, greenish blue growth; the pigment (“pyocyanin”), on which the colour depends, also diffuses through the medium.

Gelatin—liquefied.

Occurrence.—Suppurating wounds, usually in mixed infection with pyogenic cocci; otitis media, etc.

BACILLUS PROTEUS

Morphology.—Straight rods about same size as *B. pyocyaneus*; pleomorphic; motile with lateral flagella; non-sporing.

Staining.—Gram-negative.

Culture.—Grows aerobically on ordinary media. A single stroke inoculation on agar produces a moist, translucent, greyish white growth, *which tends to spread all over the available surface of the medium*—hence the designation “spreader” often applied to this organism. Gelatin and solidified serum are liquefied.

Occurrence.—Septic infections — *e.g.* suppurating wounds, urinary sepsis, otitis media, etc.—usually along with other pyogenic organisms. It occurs frequently as a saprophyte in decomposing organic matter.

Note.—In typhus fever the blood serum agglutinates specifically a *B. proteus* type (“X 19”). This reaction is utilised as a diagnostic test (“Weil-Felix reaction”), but the organism has no aetiological relationship to the disease.

BACTERIOLOGICAL DIAGNOSIS IN PYOGENIC INFECTIONS

COLLECTION OF SPECIMENS AND MICROSCOPIC EXAMINATION

Films are made on microscope slides from the pus or inflammatory exudate, dried and fixed by heat. *Pus* should be spread so as to show "thick and thin" areas. *The films should, if possible, be made direct from the lesion.* For transmission to the laboratory the material is placed into a sterile stoppered test-tube. *Drying of pus intended for examination must be avoided.* It is unsatisfactory to collect pus or discharges on gauze or cotton-wool swabs. In the case of *ulcers, sinuses*, lesions of the *cervix uteri*, etc., exudate may be taken with a sterile platinum loop, and films (and also cultures) made at once.

In the case of *pleural and peritoneal fluids*, the fluid should be withdrawn into an equal volume of sterile citrate-salt solution (sodium chloride, 0.6 per cent., sodium citrate, 1.5 per cent.). This is to avoid coagulation, which renders cytological and bacteriological examination unsatisfactory. The material is centrifugalised and films are made from the deposit.

Urine should be drawn, with aseptic precautions, by means of a catheter lubricated with sterile oil. No antiseptics are to be used. The specimen is passed directly into a sterile stoppered bottle, and should be submitted without any delay for investigation. In the female this procedure is absolutely necessary to avoid contamination. In the male, however, a fairly satisfactory specimen may be obtained by cleansing the urinary meatus and, after a portion of the urine has been voided, collecting a specimen directly in a sterile bottle. The urine is centrifugalised, and films are made from the deposit. After the films have been dried and fixed they should be gently washed in water to remove crystalline deposit.

Sputum should be expectorated directly into a

small sterile wide-mouth stoppered bottle. Films are made as in the case of pus.

Films are stained (1) by Gram's method; (2) with Loeffler's methylene blue.

CULTIVATION

Successive stroke inoculations are made on nutrient agar (if necessary, serum- or blood-agar) in Petri dishes or sloped in tubes (*vide* p. 82). A proportion of the growth will be represented by separate colonies, and the colony characters of the organisms can be recognised. Films are also made from colonies and stained by Gram's method. In this way the organism present can generally be identified. If a mixed growth results, single colonies can be "picked off" on to agar slopes, so that pure cultures are available for any further examination required.

If *septicaemia* or *pyaemia* is suspected, blood culture is carried out (*vide* p. 131).

ACNE BACILLUS

An organism associated with acne and regarded as the aetiological agent. It is Gram-positive, rod-shaped, and measures about 1.5μ by 0.5μ . It is markedly pleomorphic, and frequently shows a beaded appearance. It grows only under anaerobic conditions. The growths are not specially characteristic.

ORGANISMS SPECIFICALLY ASSOCIATED WITH CONJUNCTIVITIS

KOCH-WEEKS BACILLUS

Associated with acute contagious ophthalmia.

Morphology.—Short slender rods about 1μ in length; intracellular position is characteristic.

Staining.—Gram-negative.

Culture.—Only grows on media containing blood or serum; growth develops in form of minute dew-drop-like colonies.

DIPLO-BACILLUS OF MORAX

Associated with subacute or chronic conjunctivitis.

Morphology.—Rod-shaped organism measuring about 2μ by 1μ , in pairs end to end; non-motile.

Staining.—Gram-negative.

Culture.—Requires blood or serum for growth; on solidified serum growth produces liquefaction, and colonies develop "pits" or "lacunae" at the surface of the medium.

DIAGNOSIS IN CONJUNCTIVITIS

Films should be made from a loopful of conjunctival exudate. Stain with dilute carbol-fuchsin, or by Gram's method. Make cultures on blood- or serum-agar.

Note.—Other organisms found in conjunctivitis are: gonococcus, pneumococcus, staphylococci, streptococci, organisms of *B. coli* group. *Staphylococcus albus* and diphtheroid bacilli—*e.g.* *B. xerosis* (*q.v.*)—are frequent normal inhabitants of the conjunctival sac.

PNEUMOCOCCUS

The causative organism of pneumonia.

Morphology.—The typical appearance is that of a lanceolate or oval coccus in pairs with the rounded ends together; it is about 1μ in its long diameter; shows a thick capsule, which appears as an unstained zone round the organism unless positively stained by special methods (*vide* p. 107). In culture it is not so typical, being less lanceolate and more rounded; the capsule is not usually evident, and the cocci may occur in chains.

Staining.—Gram-positive.

Culture.—Aerobe; optimum temperature, about $37.5^{\circ}\text{C}.$; does not grow below $25^{\circ}\text{C}.$; grows on ordinary media, but best in the presence of blood or serum.

On agar—growth consists of small (about 1 mm. diameter), delicate, semi-transparent, dewdrop-like

colonies, which tend to remain discrete—*i.e.* like colonies of streptococci.

In bouillon—growth is at first a uniform turbidity, but later forms a granular deposit in the tube.

On blood agar—it is non-haemolytic and the colonies are green, due to methaemoglobin production—*i.e.* like the “viridans” types of streptococci.

Biochemical Reactions.—Ferments various carbohydrates and differs from most strains of streptococci by its fermentation of inulin. It is sharply differentiated from the streptococci by its solubility in bile. This test is carried out by adding 1 part of sterile ox bile or 1 part of a sterilised 10 per cent. solution of sodium taurocholate in normal saline to 10 parts of a bouillon culture. Rapid lysis occurs.

Note.—Serum must not be present in the cultures used for this test, as it inhibits the reaction.

Experimental Inoculation.—Pathological material containing pneumococci (*e.g.* pneumonic sputum) or a virulent culture, injected subcutaneously into rabbits or mice, produces a rapidly developing septicaemia, fatal in twenty-four to forty-eight hours; at autopsy, typical capsulated diplococci are present in large numbers in the heart blood.

Serological Types.—Four main types, classified I, II, III, IV, have been recognised, differing in their agglutination reactions. Types I, II and III are sharply defined from each other, and identified by their agglutination with specific type antisera. Pneumococci, which are not agglutinated by either Type I, II or III sera, are all grouped together as Type IV. Type III is easily recognised also by its cultural characters; it is the *Pneumococcus mucosus*, and produces raised mucoid or slimy colonies. Types I and II are the so-called “epidemic” types, being responsible for over 60 per cent. of cases of acute lobar pneumonia. Type IV consist for the most part of individual strains which are not interrelated, and the pneumococci normally occurring in the mouth and

throat as commensals belong to this type. The determination of the particular type of pneumococcus is important from the therapeutic aspect, as an antiserum (horse) prepared from Type I pneumococcus has a beneficial effect in infections due to this type.

Type II and III antisera, on the other hand, are non-effective therapeutically, even against the homologous types.

The identification of the type is carried out by using a pure culture of the pneumococcus, and known type sera (I, II, III). For ease and rapidity of obtaining a suspension of the organism, advantage is taken of the extreme susceptibility of the white mouse (*vide supra*). The animal is inoculated intraperitoneally with 0.5–1 c.c. of a saline emulsion of sputum (or any other pneumococcal exudate). The mouse may die in seven to twenty-four hours after injection; the peritoneal cavity is then opened with aseptic precautions, and the exudate is examined microscopically. If abundant pneumococci are present, the peritoneal cavity is washed out with 5 c.c. sterile saline solution and cultures are made on blood- or serum-agar plates. The peritoneal washings are centrifuged at low speed to deposit fibrin and cells. The supernatant fluid is removed and centrifuged at high speed to throw down the organisms. The bacterial sediment is then emulsified in saline solution to form a moderately dense suspension. The test is carried out as follows¹:—

Tube 1.	0.5 c.c.	Serum Type I (1:20)	+ 0.5 c.c. bacterial suspension
Tube 2.	0.5 c.c.	„ Type II (undiluted)	+ 0.5 c.c. bacterial suspension
Tube 3.	0.5 c.c.	„ Type II (1:20)	+ 0.5 c.c. bacterial suspension
Tube 4.	0.5 c.c.	„ Type III (1:5)	+ 0.5 c.c. bacterial suspension
Tube 5.	0.1 c.c.	Sterile ox bile	+ 0.4 c.c. bacterial suspension

Tube 5, containing bile *plus* bacterial suspension, determines the bile solubility of the strain and excludes streptococci. The tubes are incubated for one hour, and those showing agglutination after that time are noted.

¹ See technique of agglutination tests (p. 134).

The following table shows the results of determining pneumococcus types by this method :—

Pneumococcus Suspension 0·5 c.c.	Serum I (1:20) 0·5 c.c.	Serum II (undiluted) 0·5 c.c.	Serum II (1:20) 0·5 c.c.	Serum III (1:5) 0·5 c.c.	Sterile ox bile 0·1 c.c. + pneumo- coccus suspension 0·4 c.c.
Type I . . .	++	-	-	-	+
Type II . . .	-	++	++	-	+
Type II (atypical)	-	+	-	-	+
Type III . . .	-	-	-	++	+
Type IV . . .	-	-	-	-	+
Streptococcus .	-	-	-	-	-

+ indicates the degree of agglutination, and in column 5, bile solubility.

It is to be noted that atypical Type II organisms are only agglutinated by undiluted Type II serum. A Type IV pneumococcus shows no agglutination with any of the sera, but is bile-soluble, whereas streptococci are neither agglutinable nor bile-soluble.

Occurrence.—In lobar pneumonia pneumococci are present in large numbers in the consolidated areas, and can easily be detected in the sputum. They can be demonstrated in the blood by blood culture, and also occur in the pathological complications of pneumonia—*e.g.* pleurisy, empyema, endocarditis, pericarditis, meningitis, etc.

The pneumococcus is found in broncho-pneumonia, simple catarrhal conditions of the throat and respiratory tract, conjunctivitis, otitis media, meningitis, primary peritonitis.

As noted above, this organism may be present normally in the mouth and throat secretions.

DIAGNOSIS

A specimen of sputum is obtained (*vide* p. 166), and films are stained by Gram's method. Cultures are

made by successive strokes on a blood-agar plate. The characteristic colonies can be recognised among the other organisms that constantly develop from sputum, and can be picked off on to blood- or serum-agar slopes for purposes of isolation. The fermentation of inulin and bile-solubility are determined with the pure cultures obtained.

The occurrence of septicaemia with characteristic diplococci in the heart blood on inoculation of a white mouse with the sputum or the culture isolated, is conclusive proof of the identity of the organism present, even if the diplococci noted in the specimen or cultures are not entirely typical.

The serological identity can be determined as above.

MENINGOCOCCUS

Causative organism of epidemic cerebro-spinal meningitis.

Morphology.—Diplococcus with opposed surfaces flattened; sometimes in tetrads; cocci are about 1μ in diameter. In cerebro-spinal fluid the intracellular position in polymorph leucocytes is characteristic. In culture, involution forms are noted.

Staining.—Gram-negative.

Culture.—Aerobic conditions are necessary for satisfactory growth; temperature range is 25° – 42° C., and the optimum is about 37.5° C.; requires blood or serum for growth—*e.g.* 5 per cent. serum agar.

Colonies on serum agar—after twenty-four hours are small greyish, transparent, circular discs about 2 mm. in diameter—*i.e.* larger than colonies of streptococci; later the centre of colony becomes more opaque and raised, while the periphery remains thin and transparent; the borders may become crenated.

Biochemical Reactions.—Can be tested by growing on peptone-water-agar slopes containing 5 per cent. serum, 1 per cent. of the particular sugar, and an indicator (*vide* p. 65).

Ferments glucose and maltose with acid production, but has no action on lactose, saccharose, or inulin.

Note.—When first cultivated artificially the meningococcus tends to die quickly in culture—*e.g.* within two or three days. In culture it persists best at incubator temperature.

Serological Types.—There are two main types (Types I and II), differentiated by agglutination reactions with antisera. These represent over 80 per cent. of all cases occurring in this country. Other less frequent serological types (*e.g.* III and IV) have also been recognised.

DIAGNOSIS

In the early stages of cerebro-spinal meningitis the organisms are present usually in considerable numbers in the cerebro-spinal fluid, and can be recognised by microscopic examination. At a later stage they may be scanty and even apparently absent.

The specimen of fluid is obtained by lumbar puncture with a special platinum-iridium needle fitted with a stylette. The patient is anaesthetised and placed on the right side with the knees drawn up and the left shoulder thrown forward. The skin over the lumbar region, the operator's hands and the needle are sterilised by appropriate methods, and a sterile stoppered tube should be ready for collection of the fluid. The puncture is made in the middle line between the 3rd and 4th or 4th and 5th lumbar vertebrae, the needle being inserted, with the stylette in position, in a forward and slightly upward direction. In the adult it is introduced to a depth of about 5–6 c.m. The stylette is withdrawn, and the fluid is allowed to flow into the test-tube. In a case of cerebro-spinal meningitis the spinal fluid is under pressure and turbid in appearance, due to the large number of pus cells present.

In the laboratory the fluid is centrifugalised, and films are made from the sediment and stained by (1)

methylene blue, (2) Gram's method. Cultures should also be made on blood- or serum-agar. Films are made from the resulting colonies or growth and stained by Gram's method. The colony characters should be ascertained, and subcultures for further tests are obtained by picking off single colonies on blood- or serum-agar slopes. The biochemical reactions can be tested and, if necessary, the serological group identified by agglutination tests with antisera to the various recognised types.

For clinical diagnosis the microscopic examination is generally sufficient—*i.e.* if Gram-negative, intracellular diplococci with the characteristic shape of the meningococcus are observed.

In carrying out routine investigations of cases of meningitis, other causal organisms must be considered—*e.g.* pneumococcus, tubercle bacillus, etc. If no organisms are detectable in films stained by methylene blue or Gram, a Ziehl-Neelsen preparation should be examined for tubercle bacilli (*vide* p. 102). In tuberculosis the cell exudate is mainly lymphocytic as compared with the polymorph exudate in meningococcal and pneumococcal meningitis. It has also to be noted that the virus of poliomyelitis may produce inflammatory conditions of the meninges, indicated by the presence of a leucocytic exudate in the cerebro-spinal fluid. In such cases no visible organisms are present.

DIAGNOSIS OF MENINGOCOCCUS CARRIERS

During an epidemic of cerebro-spinal meningitis healthy contacts may become carriers, and meningococci are found in the naso-pharynx. The recognition of such carriers is of great importance in controlling the spread of the disease, and this depends on culturing the meningococcus from the naso-pharyngeal secretion.

Cultures should not be made within an hour after a meal, or within twenty-four hours after the application of antiseptics to the throat.

The specimen is obtained by means of a throat swab (*vide* p. 181), with the terminal $\frac{3}{4}$ -in., carrying the cotton-wool pledget, bent through an angle of about forty degrees. It is necessary that cultures should be made immediately after swabbing and the medium incubated at once, owing to the feeble viability of the organism apart from the body.

The tongue is depressed, and the swab is passed behind the soft palate and introduced into both posterior nares. Before withdrawing, the swab is also rubbed over the posterior wall of the naso-pharynx. The swab must be introduced and removed from the mouth without touching the tongue. The patient should be asked to phonate during the swabbing.

The swab is rubbed at once over a small area at the edge of a serum- or blood-agar plate already prepared, and then successive stroke inoculations are made on the remainder of the plate by means of a platinum loop, the loop being charged several times from the area inoculated directly with the swab. The plate must be incubated without delay.

Suspicious colonies are examined by means of Gram-stained films, and subcultures from single colonies are made on serum- or blood-agar slopes.

The resulting pure cultures are then available for identification. The differentiation of other Gram-negative throat diplococci from the meningococcus is considered below.

The final identification is carried out by means of a polyvalent agglutinating anti-meningococcus serum or mono-type sera to the different serological types.

THE GRAM-NEGATIVE DIPLOCOCCI OCCURRING AS NOSE AND THROAT COMMENSALS

MICROCOCCUS CATARRHALIS

A frequent commensal in the throat and nose, and generally regarded as pathogenic in catarrhal inflammations of the respiratory tract.

Morphology and Staining.—Practically identical with meningococcus.

Culture.—Grows on ordinary media without serum; grows even at room temperature: the colonies are larger than those of meningococcus, thicker and more opaque, and exhibit a somewhat tough consistence, so that they can actually be moved about on the medium with a platinum loop without being broken up. The biochemical reactions are shown in the Table (*vide infra*).

This organism is not agglutinated by meningococcus antisera.

MICROCOCCUS FLAVUS TYPES

The *morphology* of these organisms is like that of *M. catarrhalis*, and they grow on ordinary media at room temperature. Cultures develop, after forty-eight hours, greenish yellow or greenish grey colours.

Note.—Young colonies may closely simulate those of the meningococcus.

Biochemical reactions are shown below in the Table.

They are not agglutinated specifically by meningococcus antisera, but may agglutinate in low dilutions of horse serum. This has to be remembered in identifying meningococci from carriers.

MICROCOCCUS PHARYNGIS SICCUS

Resembles *M. catarrhalis*, but its colonies are markedly dry, tough and adherent to the medium.

Biochemical Reactions.—See Table.

DIPLOCOCCUS CRASSUS

Resembles *M. catarrhalis*, but shows marked variation in its staining by Gram's method, many individual cells staining Gram-positive. The colonies are small and rather like those of the streptococci.

Biochemical Reactions.—See Table.

FERMENTATIVE REACTIONS OF GRAM-NEGATIVE DIPLOCOCCI

	Glucose	Lactose	Saccharose	Maltose
Meningococcus .	⊥	-	-	⊥
Gonococcus .	⊥	-	-	-
M. catarrhalis .	-	-	-	-
M. flavus types .	⊥	-	(⊥)	⊥
M. pharyngis siccus .	⊥	-	⊥	⊥
D. crassus .	⊥	⊥	⊥	⊥

(⊥ = acid ; ± = variation in reaction among different types)

GONOCOCCUS

The causative organism of gonorrhoea.

Morphology.—Diplococci with opposed surfaces flattened or even concave. The diameter of the coccus is about 1μ . In inflammatory exudate the intracellular position of the organism is characteristic, and pus cells may often appear to be almost filled with ingested diplococci. In culture involution forms are frequent.

Morphologically the gonococcus is identical with the meningococcus.

Staining.—Gram-negative.

Culture.—Aerobe ; temperature range— 25° – 39° C., optimum— 37.5° C. ; requires blood or serum for growth.

Colonies—are semi-transparent discs about the size of a pin's head, tending to remain discrete, circular in outline at first, but later showing a "scalloped" margin, a raised, more opaque centre, and sometimes radial and concentric markings.

Biochemical Reactions.—Can be tested for as in the case of the meningococcus ; ferments glucose, but not maltose (*vide Table supra*).

Occurrence.—In the *male* the gonococcus infects the mucosa of the urethra and produces a suppurative inflammation with purulent discharge. Gonococci are present in large numbers in the discharge at an

early stage, but later diminish, and are associated with secondary infections—*e.g.* with pyogenic cocci. They may invade the prostate, vesiculæ seminales, epididymis, bladder mucosa, peri-urethral tissue (producing a peri-urethral abscess).

In the *female* the urethra and cervix uteri are infected, but rarely the vaginal mucosa. Bartholin's glands, the endometrium, and the Fallopian tubes may be invaded, and even the peritoneum.

Purulent conjunctivitis may occur as a complication. Blood invasion may result from primary gonorrhoeal infections, and arthritis is a common complication.

In female infants and children, gonococci may produce a persistent vulvo-vaginitis.

In new-born infants gonorrhoeal ophthalmia may result from direct infection at birth.

DIAGNOSIS

Films are made from the discharge.—In the *male*: from the urethral discharge; the meatus should be cleansed with sterile gauze and specimens are taken either with a platinum loop, or directly on slides. In the *female*: from the urethra and cervix uteri, using a platinum loop and with the aid of a vaginal speculum.

The films are stained by (1) methylene blue, and (2) Gram's method, and in the acute stage, both in the male and female, the occurrence of the characteristic *Gram-negative intracellular* organisms is, for clinical purposes, diagnostic.

In *Chronic infections*, particularly in the female, gonococci may be relatively scanty in films, and difficult to identify accurately among the secondary infecting organisms. In the male the "morning drop" of secretion from the urethra should be examined, or films from the discharge after prostatic massage. In the female the secretion from the cervix uteri should be examined.

The diagnosis may be confirmed by cultivation, but where there is a mixed infection this may be technically difficult. The material to be cultivated should, if possible, be made directly from the patient on blood- or serum-agar, and the culture should be incubated at once. If the material is kept at room temperature for some time before inoculation and incubation, or if it is allowed to dry, the organisms, being strict parasites, may die and fail to grow on the culture medium.

BACILLUS OF DUCREY

Associated with "Chancroid" or "Soft Sore."

Morphology.—A rod-shaped organism $1-2\mu$ by 0.5μ ; occurs in pairs and chains; present in the exudate from the sore, in the tissue lesion and in the secondary buboes; it is non-motile and non-sporing.

Staining.—Gram-negative.

Culture.—This organism has proved difficult to cultivate artificially, and appears to be a strict parasite. It has been found that primary cultures can be obtained, however, from the sore by inoculating directly tubes containing coagulated rabbit's blood. These are prepared by distributing fresh rabbit's blood withdrawn by cardiac puncture, in amounts of 1-2 c.c. in small test-tubes. When the blood has clotted, the tubes are heated at 55°C . for thirty minutes. After growth in these, the organism can be isolated on blood-agar plates. This method has been used for diagnostic purposes.

Pure cultures can also be obtained by puncturing a bubo with a syringe, drawing up some of the pus, and with this inoculating tubes of coagulated blood.

BACILLUS DIPHTHERIAE

The causative organism of diphtheria.

Morphology and Staining.—Slender rod-shaped organism, straight or slightly curved; the average size is 3μ by 0.3μ , but longer and shorter forms may be

noted; the ends are often swollen; it is non-motile and non-sporing. In culture involution forms may be observed which are pear-shaped, club-shaped, or even globular. The bacillus is Gram-positive; stained with methylene blue it shows marked "beading," and by Neisser's method (*vide* p. 105) metachromatic granules are characteristic, staining blue-black in contrast with the light brown coloration of the rest of the organism; the granules are often polar in situation.

Note.—These characteristic staining reactions may only be elicited in culture when the bacillus is grown on a serum medium.

Culture.—Aerobe; temperature range—20°–40° C., optimum—37.5° C.; grows on ordinary nutrient media, but best on serum media.

Colonies on serum media—at first very small, circular, white, opaque discs with regular borders; later the centre becomes thicker and the borders crenated; they may reach in diameter 4–5 mm. after several days' growth; sometimes the growth on serum shows a distinct yellow tint.

Does not liquefy gelatin.

In bouillon—grows in small white masses, which settle in bottom of tube and also adhere to side of tube; a surface film of growth may also develop.

Biochemical Reactions.—Ferments with acid production, glucose, maltose and dextrin, but not saccharose or mannite. These reactions can be elicited by using Hiss's serum water medium (*vide* p. 68).

Occurrence.—The bacilli are present in large numbers in the "false membrane" and in the throat secretions. They do not invade the lymphatics to any extent, and there is no general blood infection. In nasal diphtheria the organisms can be detected in the nasal discharge. Infection of wounds, the conjunctiva, vulva and vagina may occasionally occur.

Diphtheria Toxin.—The diphtheria bacillus produces an extracellular toxin with specific toxic properties.

While the bacillus remains localised at the site of infection, the diffusible toxin is absorbed into the blood stream and leads to the secondary lesions of diphtheria—*e.g.* post-diphtheritic paralysis.

The toxin is obtained artificially as follows :—

A meat extract is prepared from veal, and rendered sugar-free by adding distillery yeast and incubating for about sixteen hours ; the extract is converted into a nutrient bouillon by adding 2 per cent. Witte's peptone and 0.5 per cent. sodium chloride, and the medium is standardised to a pH of 7.4 ; a shallow layer of the medium is placed in 500 c.c. conical flasks so that a relatively large surface is exposed to the air ; it is abundantly inoculated with *B. diphtheriae*, and after eight to twelve days incubation at 37.5° C. the culture is filtered through a porcelain filter, the filtrate containing the toxin (*vide* pp. 22, 279).

By immunising animals (*e.g.* horses) with this preparation of toxin, a serum antitoxin can be developed, and is extensively used for the prophylaxis and treatment of diphtheria.

For further details of the preparation of toxin and antitoxin one of the larger works should be consulted.

DIAGNOSIS

A specimen of the throat secretion should be obtained. No antiseptics (*e.g.* in form of gargles, etc.) must have been applied within twelve hours. A sterile throat swab¹ should be rubbed over the affected area, or, where there is no definitely localised lesion, over the mucous membrane of the pharynx and tonsil. A tube of Loeffler's serum is inoculated by smearing the infected swab over the whole surface of the medium, moistening the swab in the condensation water at the

¹ A stiff wire with a pledget of cotton-wool firmly wrapped round one end for about $\frac{3}{4}$ in. The wire is kept in a narrow hard glass test-tube, and for convenience the other end is fastened to the cork which stoppers the tube. The tube with the contained wire and swab is sterilised by dry heat.

foot of the tube. The tube is incubated for eighteen to twenty-four hours at 37.5°C .

Films are made from the resulting culture and stained by (1) Loeffler's methylene blue, (2) Neisser's method, or (3) Pugh's stain.

Films may also be made directly from the swab and stained by the above methods, and in a proportion of cases positive results may be obtained immediately. Cultures are, however, generally made as a routine procedure, irrespective of direct examination.

In the case of suspected throat diphtheria, the appearance in cultures of bacilli showing the characteristic morphology and staining reactions (especially the metachromatic granules by Neisser's stain) is diagnostic.

In the case of supposed nasal diphtheria, or diphtheria affecting mucous surfaces other than the throat, or wounds, the microscopic examination of cultures is not conclusive. The suspected organism must be isolated in pure culture on serum-agar plates from the primary growth, which is usually a mixed one, and its fermentative characters and the virulence must be tested. The virulence test consists in injecting 2 c.c. of a two days bouillon culture subcutaneously into a guinea-pig (*vide* p. 124). The animal dies usually within a day or two, showing a local lesion at the site of injection and congestion of the internal organs, with characteristic enlargement, congestion and haemorrhage of the suprarenal glands. The injection of a control animal with the same dose of culture *plus* diphtheria antitoxin is an additional test. The control animal should survive.

Note.—The animal test depends on the virulence of the strain injected, and avirulent strains of diphtheria bacilli may be noted in the investigation of diphtheria carriers.

**BACTERIA BIOLOGICALLY ALLIED TO THE
DIPHTHERIA BACILLUS****BACILLUS OF HOFMANN**

A throat commensal.

Morphology and Staining.—Compared with *B. diphtheriae* it is shorter and may present a somewhat oval appearance; stained with Loeffler's methylene blue, an unstained bar in the middle of the organism is a frequent character and renders it not unlike a diplococcus. It is Gram-positive; no metachromatic granules are detected by Neisser's method.

Culture.—Grows aerobically on ordinary media; growths are more abundant than those of *B. diphtheriae*, and the colonies are larger and more opaque.

Biochemical Reactions.—See Table.

It is non-virulent to laboratory animals.

BACILLUS XEROSIS

A commensal in the conjunctival sac. Closely resembles *B. diphtheriae*, but metachromatic granules are not usually demonstrable.

Can be differentiated from *B. diphtheriae* by biochemical reactions (*vide* Table) and by its non-pathogenicity to laboratory animals.

OTHER DIPHTHEROID BACILLI

Certain of these organisms present a close similarity to *B. diphtheriae*, and may exhibit the characteristic granules by Neisser's staining method. They are mostly non-pathogenic, and have been isolated from the secretions of the nose and naso-pharynx, from the skin, lymph glands (apart from disease) and other tissues. Certain of these organisms have been regarded as pathogenic—*e.g.* in Hodgkin's disease, skin diseases, etc. Compared with the true diphtheria bacillus they are of low virulence to laboratory animals.

BIOCHEMICAL REACTIONS OF *B. DIPHTHERIAE* AND ALLIED TYPES

	Glucose	Saccharose	Dextrin
<i>B. diphtheriae</i> . . .	⊥	-	⊥
<i>B. of Hofmann</i> . . .	-	-	-
<i>B. xerosis</i> . . .	⊥	⊥	-

(⊥ = acid)

BACILLUS TUBERCULOSIS

The causative organism of tuberculosis in man and animals.

Under this designation are included different types—the “human,” “bovine” and “avian” types.

HUMAN TYPE

Morphology.—Slender straight or slightly curved rod-shaped organism, $2.5-3.5\mu$ by 0.3μ , with rounded, pointed, or sometimes swollen ends. In the tissues it may occur singly, or in pairs often forming an obtuse angle, or in small bundles of parallel bacilli. The bacillus is non-motile and non-sporing, though it possesses considerable powers of resistance to drying. In old cultures individual cells may grow out into long filaments and show branching.

Staining.—It is more difficult to stain than other bacteria. A strong dye with a mordant is required (*e.g.* carbol-fuchsin—*vide* p. 102), and either prolonged staining or the application of heat. It may stain uniformly or show marked beading. When stained it resists decolorisation with 20–25 per cent. sulphuric or nitric acid, and also with alcohol, and is therefore described as “acid- and alcohol-fast.” The tubercle bacillus is Gram-positive, but can only be demonstrated with difficulty by Gram’s method. The “acid-fastness” is due to a lipid sheath which can be dissolved out with hot alcohol or ether.

Culture.—Aerobe; temperature range— $30^{\circ}-41^{\circ}\text{C.}$, optimum— $37^{\circ}-38^{\circ}\text{C.}$

Will not grow on ordinary media. Primary growths may be obtained on serum media, on pieces of animal tissue, or on a medium containing egg yolk (*vide* p. 71). In secondary culture, growths may result on ordinary media (agar, bouillon, potato) with 5–6 per cent. glycerin added; growth on culture medium is slow—*i.e.* ten days may elapse after primary inoculation, or even sub-inoculation, before growth is apparent.

The most convenient medium for artificial culture in ordinary laboratory work is glycerin-egg medium (*vide* p. 71); the growth is luxuriant and presents the following appearance:—dry, irregular, tough and tenacious, wrinkled or mammillated, at first white, later buff-coloured.

In glycerin broth—growth consists of white flocculi forming a powdery deposit, but it may spread on the surface of the medium as a white wrinkled pellicle.

Tuberculin is a preparation representing the broken-up protoplasm of the bacillus *plus* its products in artificial culture medium. It was originally prepared from a six-weeks-old culture in glycerin bouillon, evaporated to one-tenth of its volume, sterilised by heat and filtered (Koch's old tuberculin). For its uses and other varieties of tuberculin, reference should be made to one of the text-books.

Animal Inoculation.—The guinea-pig is susceptible to experimental infection. If injected subcutaneously with the bacilli either in pathological material or in culture, after a few days a local swelling results consisting of tubercle nodules, which become confluent, undergo caseation and finally ulcerate. The neighbouring lymph glands become involved by spread of the bacilli along lymphatic channels and, later, lymph glands in other parts of the body are affected, showing the characteristic tuberculous lesions. The animal begins to lose weight, and dies in six weeks to three months. At autopsy, a general tuberculosis is noted; the spleen is enlarged and contains greyish white tuberculous nodules or larger necrotic lesions. The

liver and lungs present a similar condition, and also various other organs.

Animals can also be infected by inhalation and by feeding.

BOVINE TYPE

Morphology and *Staining* reactions are practically identical with those of the human type.

Culture.—As compared with the human type (“eugonic”), growth is less luxuriant, and the bovine type is described as “dysgonic.” On egg medium—it forms a thin, white, smooth, slightly moist, granular and easily broken-up growth (*cp.* human type). The difference between the human and bovine types is accentuated by using a glycerin-egg medium.

Pathogenicity to Animals.—The bovine type is more virulent to bovines and laboratory animals than the human type. In the ox it produces a fatal tuberculosis, whereas the human type causes only a localised lesion, which heals spontaneously.

The difference between the two types can be elicited by injecting a rabbit intravenously with an emulsion of 0.1 mgm. of dried bacilli (from a culture) in saline. The bovine type produces an acute generalised tuberculosis, and the animals usually die within two months; in the case of the human type the animals survive, or die only after two months.

It is to be noted that types intermediate in character between the human and bovine types have been found.

Occurrence of Tubercle Bacilli in Human Lesions.—Both the human and bovine types are met with. The latter is rarely found in adults, but a proportion of tuberculous conditions in young subjects are due to this type, particularly cases resulting from alimentary infection by milk from tuberculous cows.

Tubercle bacilli are most numerous in acute lesions showing rapid cascation—*e.g.* acute phthisis. In acute miliary tuberculosis they are relatively scanty. In chronic infections few tubercle bacilli are present, and

they may not be detectable microscopically though demonstrable by animal inoculation—*e.g.* in the pus from a tuberculous abscess. In the lesions they are usually found free from cells, but intracellular bacilli may be noted.

In phthisis, tubercle bacilli are present in the sputum, and often in large numbers if the pulmonary lesion is active and rapidly breaking down.

In tuberculosis of the urinary system the bacilli may be found in the urine by microscopic examination of the deposit after centrifugalisation, but as a general rule they are relatively scanty, and may not be observed in films.

In intestinal tuberculosis the bacilli may in some cases be noted in films from the faeces.

In tuberculous meningitis, tubercle bacilli may be seen in films from the fluid after centrifugalisation, but in early cases microscopic examination may yield negative results.

DIAGNOSIS

In general, microscopic examination is sufficient for ordinary diagnostic work.

Sputum—a film is prepared from the purulent portion of the sputum and stained by the Ziehl-Neelsen method (*vide* p. 102). A prolonged examination may be necessary in some cases where the bacilli are relatively scanty, and one examination with a negative result by no means excludes tuberculosis.

Urine, pleural, peritoneal and cerebro-spinal fluid—are centrifugalised, films are made from the deposit and stained by the Ziehl-Neelsen method. In the case of specimens of urine not taken with a catheter, it is absolutely essential to treat the film with alcohol (two minutes) after decolorisation with acid in the Ziehl-Neelsen process, in order to eliminate smegma bacilli (*vide* p. 104).

Pus and faeces—films are made in the usual way.

In the case of tissues—sections are cut, and stained by the Ziehl-Neelsen method (*vide* p. 104).

Antiformin method for the detection of scanty tubercle bacilli in sputum, pus, tissues, etc.—

By this method the bacilli can be concentrated in the material examined, and it is particularly valuable where the bacilli are scanty. The method is of course entirely supplementary to the ordinary examination, and is quite unnecessary where bacilli can be seen in direct film.

“Antiformin” consists of equal parts of liquor sodae chlorinatae (B.P.) and 15 per cent. caustic soda. It has the property of dissolving cells and other bacteria, leaving acid-fast organisms intact.

A quantity of sputum or other material is treated with three or four times its bulk of antiformin diluted 1 in 6 with water, and the mixture is shaken and allowed to stand at 37·5° C. till it becomes thoroughly liquefied. This usually takes about an hour, but it is sometimes necessary to add more diluted antiformin to complete the solution. The mixture is then centrifugalised and the supernatant fluid is removed. Water is added, and mixed with the sediment. After centrifugalisation again, thick smears are made from the sediment, dried, fixed and stained by the Ziehl-Neelsen method.

Cultivation of Tubercle Bacilli from Pathological Material.—If the tubercle bacilli are likely to be present in pure culture in the material, tubes of Dorset's egg medium can be inoculated directly. If other organisms are present—*e.g.* in sputum—the antiformin method can be used and cultures made from the sediment; in this case the centrifuge tubes, water used for washing the sediment, etc., must be sterile. A slower but more certain method of obtaining pure cultures is to inoculate a guinea-pig with the material, and to make cultures from the lesions in internal organs (*e.g.* spleen) after generalised tuberculosis results.

In laboratory diagnosis, where tubercle bacilli cannot be detected in specimens by microscopic examination, the *animal inoculation test* (guinea-pig) may be

resorted to, and it often yields positive results when microscopic examination is negative.

AVIAN TUBERCLE BACILLUS.—The causative organism of a tubercle-like disease in birds. Its morphology and staining reactions are the same as in the case of the other types of tubercle bacilli. Its optimum temperature is 43.5°C ., and on glycerin-agar the growth is more rapid in development and more luxuriant and moister than that of the mammalian types.

It is highly virulent to fowls which are resistant to the mammalian tubercle bacilli.

Acid-Fast Bacilli found in Cold-Blooded Animals.—Acid-fast bacilli resembling the tubercle bacillus have been isolated from fish, frogs, turtles, etc., and have been regarded as associated with a tubercle-like disease in such animals. These organisms grow at low temperatures, even 15°C .

Acid-Fast Saprophytic Bacilli.—Non-pathogenic acid-fast bacilli may be found in milk, butter, manure, grass. They are similar in morphology to the tubercle bacillus, but their growth on culture medium is rapid; they grow on ordinary media and at room temperature.

SMEGMA BACILLUS.—This is a commensal organism found in the smegma and on the skin. As it may occur in specimens of urine, it has to be carefully differentiated from the tubercle bacillus. It is generally shorter and thicker than the latter, and shows greater variation in size and shape. The smegma bacillus is acid-fast, but is decolorised by alcohol (*vide* p. 104), which has no effect on the tubercle bacillus. It grows readily on ordinary media, and is practically non-pathogenic to animals.

BACILLUS LEPRÆ

The causative organism of leprosy.

Morphology and Staining.—A straight or slightly curved slender bacillus, about the same size as the

tubercle bacillus, with pointed, rounded, or club-shaped ends; as far as is known it is non-motile and non-sporing. Like the tubercle bacillus it requires as a rule a strong stain, and is acid-fast, though not to the same extent; it may stain uniformly, but usually shows marked beading, which is somewhat coarser than that of the tubercle bacillus; it is Gram-positive, and can be stained fairly readily by Gram's method.

Culture.—A great many attempts have been made by various workers to cultivate this organism; the majority have been unsuccessful, and where successful results have been claimed and cultures of acid-fast bacilli have apparently been isolated from leprosy lesions, the question is still an open one as to whether these strains represent the true leprosy bacillus. The attempted cultivation of this organism is hardly within the scope of routine practical bacteriology, and for further information on this subject the text-books and other literature should be consulted.

Occurrence and Distribution.—Leprosy is an infective granuloma, developing as (1) the "nodular" type, in which nodules of granulation tissue form in the skin, mucous membranes and internal organs, or (2) the "maculo-anaesthetic" type, where the granulation tissue infiltrates certain nerves and leads to motor and sensory paralysis with characteristic trophic changes (*e.g.* anaesthetic skin areas—"maculae").

The bacilli are found in the granulomatous lesions, being particularly numerous in the nodular form. They are distributed intracellularly for the most part, lying in bundles of parallel bacilli, and may completely fill up cells. Leprosy bacilli may also be found in the tissue spaces, in the walls of small vessels, in skin glands, lymph glands, and in the secretions of the nose, throat and mouth, due to the fact that the mucosal lesions ulcerate readily and discharge bacilli into the mucous secretions. The bacilli are not found, of course, in the maculae, which are essentially trophic, and not primary leprosy lesions. In leprosy the bacilli

have actually been found in organs without associated lesions.

The bacilli are less numerous in the nerve granulomata than in the nodular lesions.

DIAGNOSIS

Films are made from any ulcerated nodule on the skin, or a non-ulcerated nodule can be punctured with a needle and squeezed till lymph exudes, from which films are made. Films can also be prepared from a scraping of an excised piece of tissue, or sections may be prepared as for histological examination.

The films or sections are stained by the Ziehl-Neelsen method, substituting 5 per cent. sulphuric acid for 20 per cent. The presence of the characteristic acid-fast bacilli, especially when they occur in large numbers and are situated inside cells, is diagnostic.

As a routine measure, films should be made in all cases from the nasal secretion, as diagnostic information may be obtained in this way even when nodules are not present in the skin. This also applies to the maculo-anaesthetic cases.

BACILLUS MALLEI

The causative organism of glanders.

Morphology.—Straight or slightly curved bacilli with rounded ends, about $3-5\mu$ by 0.5μ . Shorter forms are frequently noted and also longer filaments. Bacilli with club-shaped ends, and even branched forms have been observed. In old cultures swollen irregular involution forms are numerous. The bacilli occur singly or in pairs. They are non-motile and non-sporing.

Staining.—Gram-negative. An important feature of the organism, as seen in the tissues and inflammatory exudate, is its granular or beaded appearance.

Culture.—Aerobe; optimum temperature — 35° – 38° C.; does not grow below 25° C.; grows on ordinary

media, but the addition of glycerin (5 per cent.) assists growth.

Agar stroke—uniform white semi-transparent moist band of growth along needle track, which later becomes opaque, somewhat slimy and yellowish brown in colour. This growth is not specially characteristic.

Potato slope—at first a transparent yellowish growth (“honey-like”), later becoming opaque and of a chocolate-brown colour.

In primary culture, growth does not occur readily and the organisms die out quickly, but after several subcultures they become adapted to the saprophytic existence, and may live in culture for two to three months.

Note.—*Mallein* is a preparation analogous to tuberculin. For its preparation and applications reference should be made to the text-books.

Occurrence.—Glanders is an infective granuloma, with a marked tendency, however, to suppurative change. It is essentially a disease of horses, asses and mules, and is only occasionally transmitted to man, usually by direct infection from an animal source.

In acute and subacute animal glanders, ulcerating nodules occur in the nasal mucosa and later in the lungs and internal organs. The bacilli are present in considerable numbers in all the lesions, situated for the most part extracellularly.

In chronic animal glanders (“Farcy”), where the superficial lymph gland and vessels are involved, the bacilli are less numerous.

“Latent” infections are not infrequent in animals.

In human glanders, the infection usually originates in the skin (*e.g.* wound, abrasion, etc.), more rarely in the mucosa of the mouth or nose. The bacilli are found in the local inflammatory lesion and spread by the lymphatics, producing an acute lymphangitis. Ultimately a pyaemic condition results with secondary foci, in which the bacilli are numerous.

Animal Inoculation.—In the equidae, the typical disease can be reproduced by subcutaneous injection of recently isolated cultures; asses are most susceptible, and can be utilised in special cases for diagnostic inoculation. Guinea-pigs are markedly susceptible, and after subcutaneous injection die in a week or two with generalised lesions, as in acute animal glanders. If a male guinea-pig is inoculated intraperitoneally, the tunica vaginalis is rapidly invaded, and externally swelling of the testis is noted. Even when pathological material containing the bacillus mallei in mixed infection is injected intraperitoneally, the glanders organism seems to flourish best in the tunica, and can be more easily isolated from this situation than from the peritoneum.

DIAGNOSIS

Films are prepared from the pus, discharge from sores, etc., or from nodules in internal organs noted at post-mortem; these are stained with methylene blue and Gram's method. The appearance of beaded Gram-negative organisms corresponding to *B. mallei* is suggestive.

Cultures are also made on glycerin-agar, and if a mixed growth results, pure growths are obtained by subculturing single colonies. The chocolate-coloured growth on potato is an important criterion in identification.

In all cases the nature of the infection must be confirmed by animal inoculation. A male guinea-pig is injected intraperitoneally with the pathological material or the culture isolated; in two or three days an enlargement of the testis results, and the animal subsequently dies, showing the lesions of acute glanders (*vide supra*). If pyogenic organisms are also present in the material injected, and a septic peritonitis results, the glanders bacilli will be found more numerous in the tunica vaginalis.

BACILLUS ANTHRACIS

The causative organism of anthrax in animals and man.

Morphology.—A non-motile, straight, rod-shaped, sporing bacterium, rectangular in shape and of relatively large size— $6-8\mu$ by $1-1.5\mu$. The bacilli tend to be arranged in chains end to end (streptobacillus), but may occur singly and in pairs; in blood and tissue they exhibit a distinct capsule. The spore develops as a refractile dot in the centre of the bacillus, increasing in size till it appears as an oval structure, central in position and of the same cross-diameter as that of the bacillus. Sporulation occurs readily when the organism leaves the body of an infected animal, and spores are a morphological feature of the bacilli when growing in artificial culture, but *sporulation does not occur in the tissues*. After the spore is fully formed the residual protoplasm of the bacillus disintegrates and the spore is left as a free structure. The spore represents a highly resistant phase in the life-history of the organism, and can survive under conditions which would be unfavourable to the vegetative form. When replaced in favourable conditions, the spore capsule is absorbed and the vegetative phase is reproduced.

Staining.—Gram-positive. The spore is unstained by the ordinary methods, but can be differentially stained by special methods (*vide* p. 107).

Methylene blue reaction of M'Fadyean—this staining reaction has been utilised in veterinary work for the recognition of anthrax bacilli in blood films. The films are made in the usual way on slides, dried and passed rapidly three times through the flame; they are then stained with polychrome methylene blue (*vide* p. 96), washed and dried. Between the bacteria an amorphous purplish material is noted, representing the disintegrated capsules of the organisms; this appearance is characteristic of the anthrax bacillus.

Culture.—Aerobe and facultative anaerobe; tem-

perature range— 14° – 43° C., optimum— 35° – 37° C.; aerobic conditions are necessary for sporulation; the optimum temperature for sporulation is about 30° C.; grows on all ordinary media.

Colonies on agar—white, granular, circular discs which, under the low power of the microscope, show a wavy margin, often likened to locks of hair. The colony is one continuous convoluted thread of bacilli in chain formation.

Agar stroke—thick, white, opaque, somewhat dry, friable growth with notched edges, showing the same microscopic characters as the colonies. To the naked eye this growth presents a “ground-glass” appearance.

Gelatin stab—a line of growth along needle puncture, from which fine lateral spikes radiate—longest towards the top. This gives the so-called “inverted fir-tree growth”; later liquefaction occurs, starting at the top of the growth.

In bouillon—growth develops as white flakes, which sediment.

Occurrence in Animal Lesions.—The anthrax bacillus produces an epizootic disease in herbivorous animals, particularly among sheep and cattle. The condition is septicaemic in nature, and post-mortem the bacilli are found in large numbers in the heart blood and internal organs, especially in the spleen, which is enlarged, soft and diffuent.

Occurrence in Human Lesions.—Transmission of the infection to man is from an animal source:

- (1) Infection may occur through the skin—*e.g.* in hide porters, from shaving brushes, etc.—the resulting lesion being described as a “malignant pustule”—*i.e.* an area of intense inflammation, with the formation of a central slough and with surrounding inflammatory oedema. Lymphatic spread may occur, and even septicaemia.
- (2) Infection may result from inhalation of anthrax spores carried in dust or filaments of wool from

infected animals, as in the wool factories—"wool-sorters' disease." The organisms settle in the lower part of the trachea or in a large bronchus, and an intense inflammatory lesion results, with haemorrhage, oedema, spread to the thoracic glands, involvement of the lungs, and effusion in the pericardial and pleural cavities; the organisms are present in considerable numbers in the lesions; a septicaemic condition may also supervene.

- (3) Infection may occur by the intestine, but this is relatively uncommon in man.

Note.—Selavo's anti-anthrax serum is used for the treatment of human anthrax.

Experimental Inoculation.—Guinea-pigs and white mice are most susceptible. If a guinea-pig is injected subcutaneously with pathological material containing the bacilli, or with artificial cultures, the animal dies usually within two days, showing a marked inflammatory lesion at the site of inoculation and extensive gelatinous oedema in the subcutaneous tissues. Large numbers of bacilli are present in the local lesion. The animal shows a profound septicaemia, and the anthrax bacilli are present in large numbers in the heart blood and in the capillaries of internal organs. They are specially numerous in the spleen, which is enlarged and soft.

DIAGNOSIS

Malignant Pustule—

1. Films are made from the exudate and stained by Gram's method; the finding of anthrax-like bacilli is suggestive but not conclusive.

2. Successive stroke inoculations should be made on an agar plate. The resulting colonies are recognised by examining them with the low power of the microscope, and films are made and stained by Gram's method. Spores are noted in cultures.

3. In all cases the identity of the suspected organism should be confirmed by inoculation of a guinea-pig or mouse with exudate from the lesion, or with the isolated culture. The occurrence of the bacilli in the heart blood in considerable numbers, and the other post-mortem appearances described above are diagnostic.

Diagnosis of Anthrax in Animals.—A blood film should be made from the ear and stained by Gram's method and by M'Fadyean's methylene blue method (*vide supra*). The finding of characteristic bacilli in the blood giving the methylene blue reaction is practically diagnostic. If necessary, the organism can be cultured and identified by the procedure described above, using a specimen of blood from the ear for the investigation.

THE AEROBIC SPORING BACILLI BIOLOGICALLY ALLIED TO *B. ANTHRACIS*

These organisms are saprophytes and represent a number of different species. They are found in soil, water, dust and air. Being ubiquitous, they are frequent contaminants of culture medium in the laboratory, and bacteriological workers must be acquainted with their biological characters.

BACILLUS MYCOIDES

A common soil organism.

Morphology and Staining.—Similar to *B. anthracis*.

Culture.—Optimum temperature about 18° C. The colonies can be differentiated from those of *B. anthracis* by the long irregular threads that develop from the central growth, giving them a "spiky" or "feathery" appearance.

In bouillon — a pellicle of growth forms on the surface.

B. mycoides is quite non-virulent to laboratory animals.

B. SUBTILIS AND ALLIED TYPES

(*e.g.* B. VULGATUS, B. MESENTERICUS, B. MEGATHERIUM)

Morphology.—They tend to resemble the anthrax bacillus, but some are motile (*e.g.* B. subtilis), and certain types are shorter and have rounded ends. The spore is usually central, but types with subterminal and terminal spores occur (*e.g.* B. megatherium). In some species the spore may be wider than the diameter of the bacillus (*e.g.* B. mesentericus).

Culture.—Some species are strict aerobes (*e.g.* B. subtilis). Growth occurs at low temperatures (18°–22° C.). Growth on solid medium is dry (*e.g.* B. subtilis), gummy (*e.g.* B. mesentericus), or moist (*e.g.* B. vulgatus), and is white, greyish white, or yellowish. Some types exhibit a markedly wrinkled growth (*e.g.* B. vulgatus).

These organisms are all non-pathogenic to laboratory animals.

THE GRAM-NEGATIVE AEROBIC BACILLI OCCURRING AS COMMENSALS OR PATHOGENS IN THE INTESTINE

This class includes the following main groups :—

1. B. coli group.
2. Typhoid-paratyphoid or “ enterica ” group.¹
3. Food-poisoning group.
4. Dysentery group.

General Characters.—Gram - negative, non-sporing bacilli, about 2–4 μ by 0·5 μ (average)—aerobes and facultative anaerobes—growing best about 37·5° C.—not liquefying gelatin or solidified serum.

The various groups and species are differentiated by cultural, biochemical and serological tests.

¹ The basis of this classification is clinical rather than biological—*e.g.* the food-poisoning organisms belong biologically to the paratyphoid group.

BACILLUS COLI GROUP

This group includes a considerable number of different types, generally designated by the generic term *B. coli*, occurring normally in the large intestine of man and other mammals.

Morphology and Staining.—Gram-negative bacilli, $2-4\mu$ by 0.5μ , but filamentous forms up to $8-10\mu$ in length may occur, and short cocco-bacillary forms are not infrequent. The different types vary in motility. The motile varieties show a peritrichous arrangement of their flagella.

Culture.—Aerobe and facultative anaerobe. Temperature range $10^{\circ}-46^{\circ}\text{C.}$, optimum 37.5°C. Grows abundantly on ordinary media.

Colonies on agar—relatively large, thick, white, moist, circular discs; the opacity and size vary with different strains; some strains produce colonies and growth of a viscid consistence; the margin of the colonies may be circular or wavy.

Gelatin stab—a white line of growth develops along the needle track, with a disc at the top on the surface of the medium; gas spaces form in the medium due to the fermentation of the natural muscle sugar in the meat infusion; no liquefaction occurs.

In bouillon—a uniform turbidity.

Potato slope—white moist growth, later assuming a brownish colour.

Biochemical Reactions.—The most prevalent types of *B. coli* ("typical *B. coli*") in the intestine exhibit the following reactions:—

Motility	Glucose	Lactose	Dulcitol	Saccharose	Adonite	Inulin	Inosite	Indol	Voges and Proskauer reaction
+	+	+	+	+	—	—	—	+	—
or —			or —	or —				(vide infra)	(vide infra)

(*Vide* p. 65 for method of testing, and Table, p. 222*a*.)

The *B. coli communis* (Escherich), one of the commonest of these types, has the following characters:—

+	+	+	+	—	—	—	—	+	—
---	---	---	---	---	---	---	---	---	---

Indol Production.—This is tested for by growing the organism in peptone water, and after two days withdrawing with a sterile pipette 2 or 3 c.c. into a test-tube. An equal volume of Ehrlich's rosindol reagent is then added :

Para-dimethyl-amido-benzaldehyde .	4 grams
Absolute alcohol	380 c.c.
Pure hydrochloric acid	80 c.c.

A rose colour develops in the presence of indol, and can be separated out with amyl alcohol. The addition of a saturated solution of potassium persulphate hastens the reaction. If the indol reaction is negative after two days growth, the test should also be repeated after ten days, as some strains are slow in their production of indol.

Voges and Proskauer Reaction.—This reaction is exhibited by certain atypical varieties of *B. coli*. A strong caustic potash solution is added to a two-days glucose-bouillon culture, and the culture is then allowed to stand at room temperature for a few hours. If the reaction is positive an eosin-like colour develops, due to acetyl-methyl-carbinol. The caustic potash is added to absorb carbon dioxide.

Other Biochemical Reactions common to the typical B. coli.—On MacConkey's bile-salt neutral-red lactose agar—the colonies are rose-coloured, due to the action of the acid produced from the lactose, on the neutral red.

In litmus milk—acid and clot produced.

In media containing neutral red—a green fluorescence develops, especially under anaerobic conditions.

In media containing nitrates—nitrates reduced to nitrites.

ATYPICAL VARIETIES OF *B. COLI*

- (1) Non-gas-producing types are occasionally met with—*B. coli anaerogenes* (*vide* Table, p. 222a).
- (2) Non-indol-formers (*vide* Table).

- (3) Certain non-lactose-fermenters. Some of these may ferment lactose after spontaneous mutation in culture (*vide* Table).
- (4) "Inosite fermenters." These organisms also ferment adonite, and some exhibit the Voges and Proskauer reaction. They are also characterised by their large, raised, slimy or viscid colonies. The so-called *B. lactis aerogenes* belongs to this subgroup.

The *B. coli* Test in Water Examination is referred to on p. 151.

Occurrence of B. coli group.—Reference has already been made to their occurrence normally in the intestinal contents, where they are present in very large numbers. These organisms are also potential pathogens. They are found most frequently in pyogenic infections of the urinary tract (pyelitis, cystitis, etc.), either in pure or in mixed culture along with pyogenic cocci, cholecystitis and cholangitis, appendix abscess, peritonitis.

DIAGNOSIS

In film preparations from pus, urinary sediment, etc., stained by Gram's method, *B. coli* can be recognised as a small Gram-negative bacillus, but the morphological characters of this organism are not specific. If cultures are made on MacConkey's medium, the identity can be established at once in the case of the typical forms, by their rose-pink colonies.

It must be remembered, however, that certain strains of *B. coli*, even those which actively ferment lactose in fluid medium, may produce pale colonies on MacConkey's medium, so that for identification, it may be necessary to isolate the particular strain, and test its reactions in detail.

TYPHOID-PARATYPHOID OR "ENTERICA" GROUP

This group includes :

B. typhosus.

B. paratyphosus A.

B. paratyphosus B.

An organism closely allied to *B. paratyphosus* B has also been noted as the causative organism of certain cases of enterica, and designated *B. paratyphosus* C.

Note.—It is convenient to classify the typhoid and paratyphoid infections together as "enteric fever" or "enterica."

BACILLUS TYPHOSUS

The causative organism of typhoid fever.

Morphology and Staining.—A Gram-negative non-sporeing bacillus, about $2-4\mu$ by 0.5μ , actively motile, due to numerous long flagella (peritrichous); long filamentous forms (8μ) are common.

Culture.—Aerobe and facultative anaerobe; optimum temperature— 37.5°C . Grows well on ordinary media.

Colonies on agar—like those of *B. coli*, but smaller, thinner and more transparent; at first circular, later vine-leaf-like in outline, with a raised centre and radial ridges.

Gelatin stab—like *B. coli*, the growth is less abundant, and without gas formation. No liquefaction occurs.

Colonies on MacConkey's medium—colourless (*cp. B. coli*), *B. typhosus* being a non-lactose fermenter.

Biochemical Reactions.—See Table, p. 222a.

Occurrence.—This organism shows a selection for lymphoid tissue—*e.g.* the Peyer's patches and lymphoid follicles of the intestine, the mesenteric glands, and spleen. Thus it invades the Peyer's patches of the small intestine, and leads to an acute inflam-

matory reaction, and infiltration with mononuclear cells, necrosis, sloughing and the formation of the characteristic typhoid ulcers. It is present in large numbers in the inflamed tissue, in the ulcers, and is found in the intestinal contents and the dejecta. The mesenteric glands show similar inflammatory changes. At an early stage of the disease—during the first week or ten days—and in relapses, a condition of bacteraemia exists, and the typhoid bacillus can be demonstrated by blood culture. It is also present in large numbers in the spleen, occurring in clumps. Typhoid bacilli are frequently present in the gall bladder. They may also be excreted in the urine, sometimes producing a marked bacilluria.

The bacillus is found in other lesions occurring as complications or sequelae of typhoid fever—*e.g.* acute suppurative osteitis, abscess of the kidney, acute cholecystitis, broncho-pneumonia, ulcerative endocarditis. Even in the suppurative lesions it may be present in pure culture.

In 2 to 5 per cent. of convalescents, the typhoid bacillus persists in the body often for indefinite periods. In these carriers the bacilli are present in the gall bladder, or in foci in the kidney, and are excreted in the faeces or urine.

B. typhosus cannot exist as a saprophyte outside the body, but it can survive long enough to be transmitted by some vehicle of infection—*e.g.* sewage and polluted water, shell-fish, and vegetables contaminated with human excretal matter, house flies, articles of food contaminated by carriers, etc. Milk is a good culture medium for the typhoid bacillus, so that contamination of milk—*e.g.* by carriers—is a likely source of outbreaks of the disease.

Serological Reactions.—The serum of animals immunised with typhoid bacilli contains a specific agglutinating antibody (*vide* p. 271), and antisera are therefore employed in the identification of this species (*vide* p. 137).

BACILLUS PARATYPHOSUS A
BACILLUS PARATYPHOSUS B

Causal organisms of paratyphoid fever, which is, for all practical purposes, clinically similar to typhoid.

The morphology and general cultural characters are identical with those of *B. typhosus*.

For their biochemical reactions see Table, p. 222a. It is to be noted that biochemically the paratyphoid bacilli can be distinguished from *B. typhosus*. Biochemical differences have been recorded between the different paratyphoid bacilli (see Table), but these tests are not recommended for routine purposes. The ultimate identification is determined by agglutination reactions.

Their *occurrence* and distribution in the body of an infected person is the same as in the case of the typhoid bacillus.

B. paratyphosus A is more common in the East, whereas *B. paratyphosus* B is the commoner in Europe. In Western Europe the paratyphoid bacilli only produce a relatively small proportion of enteric illnesses.

Serological Reactions.—Antisera agglutinate specifically homologous strains, and these reactions must be utilised for routine identification.

DIAGNOSIS OF ENTERIC INFECTIONS¹

The bacteriological diagnosis depends on (1) the isolation from the body, and the identification of the bacillus, or (2) the demonstration of its presence in the body by the agglutination or Widal reaction, which is based on the occurrence of specific agglutinins to the causative organism in the serum of the infected person.

In the early stage, blood culture is the most conclusive diagnostic method, and should be employed, if possible, in all cases met with during the first seven to ten days of the illness, and in relapses (where a diagnosis has

¹ See also *Applied Bacteriology*, Browning, "Diagnosis of Enterica," by Browning, Mackie and Thornton, pp. 9-51.

not previously been established). The possibility of demonstrating enterica bacilli in the blood is much less at later stages. The method is referred to on p. 132. If the result is positive the strain is isolated in pure culture, and identified by morphological, cultural, biochemical characters (see small Table, p. 222a), and by testing it with an agglutinating antiserum to a known *B. typhosus*, *B. paratyphosus* A, or *B. paratyphosus* B, according to the biochemical reactions observed. Agglutination should occur up to approximately the end-titre of the serum (for the strain used for immunisation). Some strains, however, immediately after isolation may be inagglutinable, and it may be necessary to subculture several times and repeat the serological test.

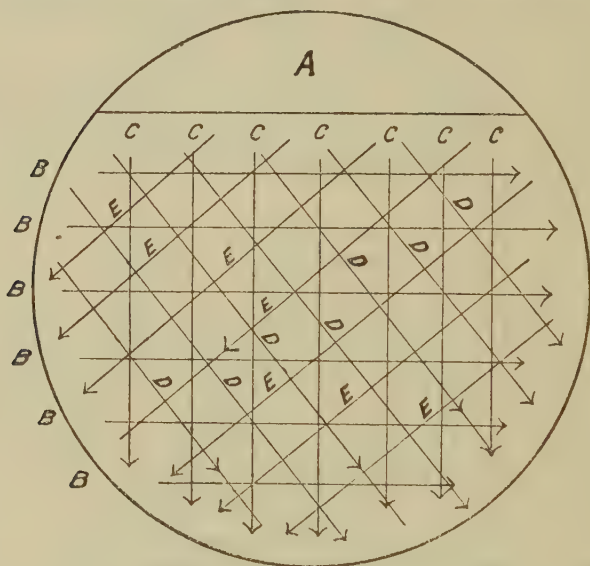
The typhoid and paratyphoid bacilli may also be isolated from faeces and urine.

FAECES.—*B. typhosus* is most frequent in the third week, *B. paratyphosus* A about the twelfth day, and *B. paratyphosus* B at the end of the second week or the beginning of the third week. Examination of faeces may frequently yield negative results unless repeated, and the isolation of these organisms from faeces is often rendered difficult owing to their being relatively scanty as compared with *B. coli*.

Direct plating of Faeces.—The medium recommended is MacConkey's bile-salt neutral-red lactose agar. It is simple in composition, easily prepared and stable as compared with other differential media for intestinal organisms. On this medium *B. typhosus* colonies are "pale" as compared with the pink colonies of *B. coli*. MacConkey's medium is also sufficiently inhibitory to the common aerial organisms to facilitate manipulation of plates in drying and inoculation. *It is essential that the surface of the medium should be thoroughly dry before inoculation.* If even a small amount of condensation water is present on the medium, a confluent growth results instead of separate colonies.

The inoculation is made by successive strokes as follows:—

A loopful of the specimen (liquid faeces or a dense emulsion in saline from solid or semi-solid faeces) is smeared over area A of the plate (see diagram). The loop is then sterilised in the flame, recharged by rubbing it over area A, and then used to inoculate the



remainder of the plate by successive parallel strokes, B, C, D and E drawn in the direction indicated by the arrow heads. The platinum wire should be held so that the whole loop is in contact with the surface of the medium. In this way the resulting colonies are evenly distributed over the plate.

This method has been found to yield highly satisfactory results. The result depends on the concentration of organisms in the specimen, and on the size of plate used. Plates of 6 in. diameter should be employed if possible. It is essential that the plates be abundantly inoculated, but if the specimen contains an

excess of organisms, it may be difficult to obtain satisfactory separation of the colonies on a small plate. The method described allows a heavy inoculation to be made with the resulting colonies well separated (except of course in area A). The plates are incubated overnight. After eighteen to twenty-four hours the colonies are usually sufficiently large for "picking off" those that are considered likely to belong to the enteric group. Some workers incubate the plates for longer periods if no suspicious colonies are noted after twenty-four hours, and occasionally this procedure is of value. The suspicious colonies can be recognised by their "pale" appearance, but other intestinal organisms may produce colourless colonies on MacConkey's medium—even strains of lactose-fermenting *B. coli* (*vide supra*). Several of these colonies are subcultured on agar slopes, and for this purpose a straight platinum wire is used.

The pure cultures isolated are tested and identified as indicated under blood culture.

The brilliant-green enrichment method for the isolation of the enteric group from faeces.—This method depends on the selective inhibition of the prevalent types of *B. coli* by brilliant green. Thus in fluid media containing certain concentrations of the dye, the typhoid-paratyphoid bacilli grow well, while the growth of *B. coli* is partially or completely restrained, and the former organisms can be enriched at the expense of the latter. This technique is more likely to yield positive results in the isolation of typhoid and paratyphoid bacilli from faeces than direct plating.

Method.—If the stool is solid or semi-solid, a dense emulsion is prepared in sterile saline solution, and from this or the liquid stool, three tubes of 10 c.c. of peptone water (2 per cent. peptone, 0.5 per cent. sodium chloride, neutral to litmus), containing the following amounts of a 1 in 10,000 watery solution of brilliant green, are inoculated:

1	2	3
0.25 c.c.	0.4 c.c.	0.7 c.c.

The 1 in 10,000 solution is made up when required from a stock 1 per cent. solution by the addition of 0.1 c.c. to 10 c.c. of sterile water, and the appropriate amounts are added to the peptone tubes.

In general a large loopful of the liquid faeces or faecal emulsion is added to the tubes. If, however, the stool is watery, three or four loopfuls should be used for the inoculation of each tube. The tubes are incubated at 37.5°C., and, after twelve hours, subcultures on MacConkey plates are made from each.

Under favourable conditions practically pure growths of the enteric bacillus may be obtained in one or more of the brilliant green tubes. Even when pure growths do not result, the typhoid or paratyphoid bacilli are relatively numerous as compared with *B. coli*, so that their isolation is easily accomplished.

URINE.—The urine is centrifugalised, and several loopfuls are inoculated on a MacConkey plate and successive strokes made in the usual way so that isolated colonies are obtained. In enterica there appear to be transient bacilluric periods, and repeated examinations of the urine are of particular value where the isolation of the causative organism is aimed at, and where other methods have been unsuccessful.

WIDAL REACTION.—The technique is described on p. 133.

Agglutinins begin to appear in the serum of the infected person *usually* about the seventh to the tenth day. Occasionally they are earlier in development (*e.g.* fifth day), but often later, and even in some cases agglutinins cannot be demonstrated at any stage of the disease. A negative result therefore may be inconclusive. The agglutination reaction, tested quantitatively, is also progressive up to a certain point—*i.e.* the end-titre of the reaction rises from the time of the first development of agglutinins in the serum. A “rising titre” is therefore highly significant in diagnosis.

Healthy carriers may also exhibit a positive agglutination reaction.

Persons inoculated with the typhoid-paratyphoid vaccine exhibit specific agglutinins in their sera, and for all practical purposes the value of the Widal reaction in such persons is thus reduced to a minimum. In cases that have been vaccinated, agglutination is of no significance unless a definitely rising titre can be demonstrated for any one of the enteric group. In the case of a person vaccinated only with *B. typhosus* and subsequently developing a paratyphoid infection, the agglutination test with the paratyphoid strain is still valid.

It must be remembered that normal serum may also agglutinate the typhoid and paratyphoid bacilli in low dilutions. The criteria of a positive reaction are—marked agglutination by the method described, in the following dilutions of serum :—

<i>B. typhosus</i>	1 in 60 or higher dilutions.
<i>B. paratyphosus</i> A	1 in 30 or higher dilutions.
<i>B. paratyphosus</i> B	1 in 120 or higher dilutions.

If agglutination occurs only in lower dilutions than these, the test should be repeated. Later results may show higher end-titres and are therefore more conclusive.

Routine Laboratory Examination of suspected Enterica Cases.—If duration less than ten days, carry out blood culture, adding 5 c.c. blood to 50 c.c. of 0·5 per cent. sodium taurocholate (supplied from the laboratory); bouillon may be used, but with the taurocholate solution there is less chance of contamination.

If blood culture negative, and when duration is more than ten days, draw blood in capsule for Widal with *B. typhosus*; if negative, take immediately another specimen for test with the paratyphoid bacilli; if this also negative, repeat serum tests in three or four days, and send specimens of faeces and urine for bacteriological examination; if no positive findings still available, repeat Widal test and examination of faeces and urine.

DIAGNOSIS OF TYPHOID AND PARATYPHOID CARRIERS

In a considerable proportion of carriers the Widal reaction is positive, and the test is of some value as

a preliminary method, but a negative result is of no significance.

The proof that a person is a carrier depends on the isolation of *B. typhosus* or *B. paratyphosus* from the faeces or urine, and at least three successive examinations should be made before the result is declared negative.

As the bacilli are likely to be most numerous in the bile and in the contents of the small intestine, three grains of calomel followed by a saline purgative should be given, and after catharsis, the second or preferably the third stool is used for the examination. The specimen should be cultured as soon as possible after it is passed. The methods used are as described above. The brilliant green enrichment method yields better results than direct plating.

The urine is examined as in the diagnosis of enterica.

BACILLUS PARATYPHOSUS C

This organism is similar to *B. paratyphosus* B, but serologically is more closely related to *B. suipestifer* (*vide infra* and Table, p. 222a).

THE FOOD-POISONING GROUP

These organisms are associated with cases of "meat or food poisoning," in which the illness takes the form of an acute gastro-enteritis with marked toxaemia. The incubation period may be short, symptoms occurring even a few hours after the ingestion of the contaminated food. Preserved meats (*e.g.* sausage) are most frequently responsible. The food may not show any obvious evidence of bacterial contamination.

Occurrence.—These organisms are found in the intestinal contents in the disease, and may produce also a bacteraemia.

As regards the source of the infection: the animal from which the flesh was taken may have been infected previous to death, or the meat may have been contaminated from some extraneous source. Organisms

of this type occur in the intestine of various animals—*e.g.* pigs. They produce epizootics in rats and other rodents. Human carriers have also been noted, and these may be responsible for contamination of foods.

BACILLUS ENTERITIDIS (GAERTNER)

This organism is similar to *B. paratyphosus B* in its various general characters (*vide* Table, p. 222*a*), but can be differentiated from it by its agglutination reactions—*i.e.* an antiserum to a known strain of *B. enteritidis* agglutinates strains of the same species, but has no action on *B. paratyphosus B*. Similarly, *B. paratyphosus B* antisera have no effect on *B. enteritidis* unless in low dilutions.

BACILLUS SUIPESTIFER

(*B. AERTRYCK*, *B. OF HOG CHOLERA*)

Similar to *B. paratyphosus B* and *B. enteritidis*, but differs in certain biochemical reactions (*vide* Table, p. 222*a*). Its differentiation from *B. paratyphosus B* by serological reactions is complicated by the occurrence of marked co-agglutination of *B. paratyphosus B.*, by *B. suipestifer* antisera, and similarly *B. paratyphosus B* antisera co-agglutinate *B. suipestifer*.

Absorption tests, however, enable differentiation to be established. If a *B. suipestifer* serum (diluted) is mixed with a dense emulsion of *B. paratyphosus B*, and after interaction for four hours at 37·5° C. the bacteria are removed from the mixture by centrifugalisation, it is found that the co-agglutinins (for *B. paratyphosus B*) have been removed from the serum, while the primary agglutinins for *B. suipestifer* are unaffected. Similar absorption of the serum with *B. suipestifer* removes both the primary agglutinins and the co-agglutinins.

Organisms, identified as *B. paratyphosus B* by general and serological characters, have been found in cases of acute gastro-enteritis following the ingestion of contaminated food.

DIAGNOSIS

The stools are plated out directly on MacConkey's medium as in enterica diagnosis, pale colonies are picked off, and the resulting cultures are tested and identified.

Blood culture may in some cases yield positive results, and should be carried out as a routine measure.

In convalescence, the serum of patients agglutinates the homologous organism, but the agglutination test is not applicable during the acute stage, as the agglutinins take some days to make their appearance in the blood.

Bacteriological examination of suspected articles of food, if available, should be carried out.

GROUP OF DYSENTERY BACILLI

The causative organisms of an acute form of dysentery most prevalent in tropical and subtropical countries, but occurring also in temperate climates—*e.g.* asylum dysentery, infantile diarrhoea.

Morphology and Staining.—Non-motile, non-sporing, Gram-negative bacilli about $2-4\mu$ by 0.5μ , but showing a tendency to shorter cocco-bacillary forms. Filamentous forms are less common.

Culture.—In cultural characters they resemble *B. typhosus*. Gelatin is not liquefied.

Biochemical Reactions.—They all ferment glucose (without gas production), and in the case of all sugar fermentations are non-gas-producing. The dysentery group can be subdivided into types according to the fermentation of lactose, dulcitol, saccharose, mannitol and maltose, the production of indol from peptone and agglutination reactions with specific antisera. The classical types are *B. dysenteriae* Shiga, *B. dysenteriae* Flexner, and *B. dysenteriae* Y (Hiss & Russell). The Flexner and Y types are so closely related serologically that, for convenience, they may be classified together and designated *B. dysenteriae* Flexner-Y.

The biochemical reactions are shown in the Table, p. 222a.

The Shiga types are also identified by their agglutination with an antiserum to a known Shiga strain.

The Flexner-Y types react with an agglutinating serum to the classical Y strain, which seems to possess antigenic properties common to both the Y and Flexner types. The Y serum is therefore used for the serological identification of the Flexner-Y type.

Besides the classical types, other dysentery bacilli are met with in tropical dysentery which do not react to a Shiga or Y antiserum, and yet have the general characters of the dysentery group. These are often designated *atypical dysentery bacilli*, and their biochemical characters are indicated in the Table. A number of types have been recognised differing according to their biochemical characters.

Occurrence.—The dysentery bacilli are found in large numbers in the stools at an early stage of the illness, even in practically pure culture, but become progressively less numerous, until, at a later stage, they are apparently absent. They do not invade the blood stream as a rule. The Shiga type is associated mostly with the acute and severe forms of dysentery, the atypical bacilli with the milder cases, and the Flexner-Y type occupies an intermediate position as regards the severity of illness with which it is associated. It may be noted here that the Shiga type, when growing under aerobic conditions, produces a powerful diffusible toxin.

Concomitants.—When dysentery bacilli tend to disappear from the stool they are found along with, or become replaced by, certain other unusual organisms which may be designated “concomitants.”

The commonest are :

B. Morgan No. 1 and allied types	} Gram-negative, non-sporing, aerobic bacilli.
B. paracolon types	
B. faecalis alkaligenes	

Their biochemical reactions are shown in the Table, p. 222a.

These organisms are also found in cases of non-dysenteric diarrhoea. Cases of *B. faecalis alkaligenes* bacteriaemia have been recorded.

B. Morgan No. 1 and allied types are responsible for some cases of infantile diarrhoea in temperate climates.

The *Diagnosis of bacillary dysentery* is dealt with later (p. 217).

THE INTESTINAL PROTOZOA

ENTAMOEBA HISTOLYTICA

The causative organism of one form of tropical dysentery.

Biology.—The vegetative forms are large, rounded, elongated or irregular amoebae, varying in diameter from 10–50 μ , the average size being about 20–30 μ . In the active condition they rapidly protrude and retract pseudopodia, and appear to move by pseudopod action, but the movements are mainly of the nature of changes in shape. The cytoplasm consists of a clear hyaline ectoplasm, and a granular, often vacuolated endoplasm. The nucleus is round or oval, and in the unstained condition is not easily distinguished. It is situated in the endoplasm, usually eccentric in position. It is poor in chromatin, and the nuclear membrane is thin. The chromatin granules are small, and are collected in a ring just inside the nuclear membrane. The amoebae ingest red corpuscles, leucocytes, tissue cells, bacteria, etc., which are observed in the endoplasm. The vegetative forms after leaving the body tend to become immobile and rounded off, and soon die and disintegrate. Multiplication is by binary fission.

Under conditions unfavourable to the amoebae—*e.g.* when the disease is becoming arrested—encystment occurs. Cysts may be detected in the stools, often in large numbers in chronic cases. After apparent recovery the patient may remain a carrier, and the encysted forms are passed in the faeces. The cyst

represents a resting-phase with increased resisting powers, and can survive outside the body for considerable periods.

Cysts are more or less spherical, with a thin hyaline refractile cyst wall, which gives them a distinct double contour. The contents are finely granular. The average diameter is 9–15 μ . The cysts contain multiple nuclei, *not more than four*, and also thick rod-shaped structures which stain deeply with haematoxylin called “chromidial bars.” These nuclei contain more chromatin than the nucleus of the vegetative form, and show a karyosome with a central chromatin dot.

The cysts are developed by division of the vegetative form into smaller “precystic” forms. The term “*Entamoeba minuta*” has been applied to these precystic forms with nuclei showing a thick ring of chromatin under the nuclear membrane.

Methods of microscopic demonstration and staining are referred to under dysentery diagnosis.

Occurrence.—In the early stage of amoebic dysentery the vegetative forms are present in considerable numbers in the large intestine and in the stools. They penetrate the mucosa of the large bowel and disintegrate the tissue by their pseudopodia and probably also by means of a liquefying ferment. The sub-mucosa is invaded, and even small veins from which the amoebae may be carried to the liver. In the bowel, oval or irregular ulcers are developed with undermined edges, which may sometimes lead to perforation of the bowel wall. There is little inflammatory reaction (*cp.* bacillary dysentery) unless a secondary septic infection occurs.

The so-called “tropical abscess” results from invasion of the liver through the portal circulation. The abscess contains a slimy chocolate-coloured “pus” consisting of necrotic tissue and blood, with only a few leucocytes or pus cells. The amoebae are found mainly in the wall of the abscess, and may not appear in the pus when first evacuated.

ENTAMOEBA COLI

A non-pathogenic intestinal amoeba which must be carefully differentiated from *E. histolytica* in diagnosis.

The vegetative forms closely resemble those of *E. histolytica*. The cytoplasm is not differentiated into endo- and ectoplasm. The nucleus is usually central in position, easily distinguishable, rich in chromatin which is often arranged in quadrant form, and has a thick refractile nuclear membrane. Amoeboid movement is sluggish. No ingested red cells are seen in the cytoplasm. The cysts are larger ($15-20\mu$) than those of *E. histolytica*, the cyst wall is thick, and there are usually *eight nuclei*, and no chromidial bodies.

ENDOLIMAX NANA

A frequent non-pathogenic intestinal amoeba. The vegetative form is 8μ in diameter or less. In unstained preparations the nucleus is not distinct, but when stained by haematoxylin it is easily demonstrated, and shows a large irregular eccentric karyosome.

The cysts are oval, and $7-9\mu$ in their longest diameter. They contain one, two, or four small nuclei, but no chromidial bodies.

IODAMOEBA BÜTSCHLI AND *DIENTAMOEBA FRAGILIS* must also be included among the intestinal amoebae of man, but need not be described here. Their characters may be ascertained by reference to recent works on protozoology.¹

INTESTINAL FLAGELLATES

These organisms are associated with dysentery and diarrhoea, but their pathogenicity is doubtful, and they may occur as commensals.

Trichomonas hominis.—It is pyriform, $9-14\mu$ long, and shows a nucleus and cytostome. It possesses

¹ See *The Intestinal Protozoa of Man*, by Dobell and O'Connor.

three flagella projecting from the broad end, and also another flagellum forming the border of an undulating membrane, and with the free part projecting from the pointed posterior end.

Chilomastix Mesnili.—Resembles *T. hominis*, but has no undulating membrane and only three flagella. It has an elongated slit-like cytostome. Cysts can easily be recognised; they are oval, about 8μ in their long diameter, and contain one nucleus.

Giardia or *Lambliia intestinalis*.—Inhabits the duodenum and jejunum.

Main characters:—somewhat flattened in shape; flat surface pear-shaped; bilaterally symmetrical; $10\text{--}18\mu$ in its long diameter; a large sucking disc on one surface; two nuclei with karyosomes; two long median parallel axostyles which represent skeletal structures, with blepharoplasts at each end; eight flagella in pairs—two arising from the anterior blepharoplasts (the broad end is spoken of as anterior), two arising near the anterior blepharoplasts but following the axostyles to the posterior edge of the sucker before diverging, two arising at the posterior edge of the sucker and rooted in the axostyles, and two arising from the posterior blepharoplasts.

The cysts are characteristic: oval in shape, about $10\text{--}15\mu$ long, with two or four nuclei, and showing the parallel axostyles.

For further information regarding *other intestinal protozoa*, reference should be made to works on protozoology.

DIAGNOSIS OF DYSENTERY

Collection of specimens of stools.—The stool should be examined as soon as possible after being passed, and should be unmixed with urine. The specimen for laboratory examination is collected in a faeces specimen tube provided with a cork carrying a metal spoon or scoop which fits into the tube, and by means of which faecal matter may conveniently be collected. In the case of a stool containing both faecal matter

and mucus, a portion of the latter should be included in the specimen.

Microscopic Examination.—A microscope slide is gently warmed over the Bunsen flame, and on one half of the slide a large drop of normal saline solution is placed and on the other a drop of Lugol's iodine.¹ A loopful of the stool or the mucous discharge is emulsified in the saline drop and another loopful in the iodine solution. The preparations are covered with No. 1 cover-slips, and examined first with the $\frac{1}{8}$ -in. and then with the oil-immersion lens.

Vegetative amoebae can usually be recognised without difficulty. In the saline preparation *Entamoeba histolytica* may often be identified by its active pseudopodial movement and the inclusion in the cytoplasm of red corpuscles (*vide supra*). On the other hand, immobile vegetative amoebae without ingested corpuscles present considerable difficulty in their identification. The presence of cysts facilitates diagnosis owing to the more striking differences between the encysted *E. histolytica* and *E. coli* (*vide supra*). In the iodine preparation the nuclei of the cysts are more distinct than in the saline preparation.

Large phagocytic body cells (macrophages) may be found in dysenteric stools, and may sometimes be mistaken for immobile amoebae by inexperienced workers. They often show vacuolation, and may even contain red corpuscles. They are, of course, immobile, and the nucleus, unless degenerate, occupying one-fourth or one-fifth of the whole cell, is relatively larger than that of an amoeba, and is not of the ring-like or vesicular type. This distinction is well seen in the iodine preparation. In a heat-fixed film these macrophage cells and their nuclei can be stained with methylene blue, while amoebae cannot be demonstrated in this way.

Other intestinal protozoa that may be present can also be detected in unstained preparations (*vide supra*).

¹ This is the iodine solution used in Jensen's modification of Gram's method, *vide* p. 100.

Where pathogenic amoebae cannot be detected, the microscopic examination often yields information of diagnostic importance.

In a case of bacillary infection there is usually an abundant and characteristic cellular exudate. The cells present are mostly pus cells with a varying number of red cells, and in the early stages, numerous epithelial cells. In addition to these, macrophages are frequently a characteristic feature of the exudate. The pus cells as a rule show marked degeneration.

In amoebic dysentery there are few body cells unless the case is also complicated by a bacillary infection. Leucocytes are scanty, and are not so degenerate as in bacillary dysentery.

The microscopic examination is therefore an important step in diagnosis: the finding of the characteristic *E. histolytica* establishes a diagnosis of amoebic dysentery, while an abundant cell exudate and the absence of amoebae would indicate bacillary dysentery.

This preliminary determination enables the bacteriologist to report at once as to the nature of the dysentery, so that the appropriate treatment can be initiated without delay.

Where no amoebae can be found, and if a diagnosis of bacillary dysentery cannot be established later, it is essential that further microscopic examinations be carried out before amoebic infection is excluded.

Stained preparations are of assistance in the identification of intestinal amoebae. Films are made on cover-glasses from the stool and are fixed "wet" by floating the cover-glasses (film downwards) in a fixing solution consisting of 2 parts saturated corrosive sublimate in saline, with 1 part absolute alcohol. They are kept in the fixing fluid for twelve to twenty-four hours and then stained with iron haematoxylin (*vide* p. 118).

Culture.—When the clinical and microscopic data point to a bacillary infection, cultures are made from the stool on MacConkey's medium as in the direct culture of enterica specimens (*vide* p. 205). *In general*

it may be said that if cases are examined within the first two or three days the infecting organism can be isolated without difficulty. At a later stage the dysentery bacilli become less numerous and "concomitants" (*vide supra*) are present often in large numbers.

For the complete bacteriological investigation of a case of bacillary dysentery therefore, it is necessary to consider all the abnormal organisms present in the stool. These are for the most part non-lactose-fermenters, and their colonies are of the "pale" type on MacConkey's medium. Certain varieties of atypical dysentery bacilli may ferment lactose in fluid medium, but in primary culture from stools present "pale" colonies after twenty-four hours.

Subcultures on agar slopes should be made from each type of pale colony on the MacConkey plate. Since colonies which appear to be similar may represent different organisms, and since there may be, in some instances, even three or four different non-lactose-fermenters present, at least four colonies should be picked off.

Sufficient growth is usually obtained after twelve hours incubation to proceed with the examination of the agar slope cultures.

Tubes of the following media are inoculated from each culture :—

- (1) peptone water
- (2) glucose peptone water (with neutral red)
- (3) lactose " "
- (4) saccharose " "
- (5) mannite " "

(*vide* p. 65)

and these are incubated for twelve hours. The peptone water culture is examined for motility after six to seven hours.

In this way one can ascertain to what group the various cultures belong, and whether two or more represent the same organism, in which case only one is kept for further examination.

If a culture is obtained with the following characters :—

	<i>Motility</i>	<i>Glucose</i>	<i>Lactose</i>	<i>Saccharose</i>	<i>Mannite</i>
Gram-negative bacillus .	-	±	-	-	- or ±

—*i.e.* corresponding to the classical dysentery bacilli, *B. dysenteriae* Shiga or Flexner-Y (see Table, p. 222a)
 —the final identification is made by tests with specific agglutinating sera—*i.e.* an antiserum to the Shiga type, and an antiserum to the classical Y strain or a polyvalent Flexner-Y serum prepared by immunising an animal with a number of different Flexner-Y strains.

If the unknown strain corresponds serologically to one of the classical types, its identification may be regarded as complete, and there is no need to investigate it further. If it fails to react to the appropriate antiserum, its biological characters should be thoroughly studied : dulcete and maltose tubes should be inoculated, and the peptone tube is tested for the presence of indol (see p. 200) after forty-eight hours, and if negative, also after seven days. The fermentation tubes should be incubated for seven days before final readings are made. A gelatin stab, or alternatively a solidified serum culture, should also be made to test for liquefaction.

If the strain still appears to have the characteristic reactions of a typical dysentery bacillus, the agglutination test should be repeated after the organism has been subcultured several times, as a small proportion of strains are inagglutinable on first isolation, but rapidly become agglutinable on subculture. If non-agglutinable, it must then be regarded as belonging to the “atypical subgroup” (see Table).

When in the preliminary tests, the sugar reactions are not those of the classical types, though the organism is a non-motile Gram-negative bacillus and does not produce gas from the sugars it ferments, it may belong to the group of atypical dysentery bacilli. Its various cultural and biochemical characters should be investigated thoroughly

as in the case of the inagglutinable strains referred to above.

The general characters of these atypical dysentery bacilli may be summed up as follows :—

Gram-negative, non-motile bacilli; not liquefying gelatin; fermenting glucose without gas; the various types differing as regards the fermentation of lactose, dulcitate, saccharose, mannite, maltose and the production of indol, but never producing gas in the fermentation of these sugars; not agglutinated by anti-Y, -Flexner or -Shiga sera even after repeated subculture; virulent to rabbits by intravenous injection.

Other abnormal intestinal organisms (*vide* p. 213) can be identified by completing the cultural tests as in the investigation of the atypical *B. dysenteriae*. Their reactions are shown in the Table.

Agglutination Tests.—In cases where the causative organism cannot be isolated from the stools, agglutination tests have a limited application in diagnosis.

Normal serum may agglutinate the dysentery bacilli in low dilutions (*B. dysenteriae* Shiga in a 1 in 50 dilution, *B. dysenteriae* Flexner-Y in a 1 in 100 dilution), and in cases of bacillary dysentery the specific agglutinating effect of the serum towards the actual causative strain isolated from the case may be relatively weak. Normal serum effects introduce, therefore, a possible fallacy in the diagnostic test.

As in the case of the Widal reaction, the agglutination reaction is only applicable five to seven days from the onset of the illness.

In general the test is carried out on the same principle as the Widal reaction (*vide* p. 134); known strains of *B. dysenteriae* Shiga and Y are tested in parallel series with varying dilutions of the patient's serum.

BACILLUS OF FRIEDLÄNDER (PNEUMOBACILLUS)

Originally described as a causative organism of pneumonia.

Morphology and Staining.—A small non-motile, non-sporing, Gram-negative bacillus with rounded

ends varying greatly in size—1 to 4μ by 0.5 to 1μ . The shorter forms simulate cocci. The bacilli occur usually in pairs, but also singly, and in short chains. They are typically capsulate, especially when seen in the tissues.

Culture.—In cultural characters it resembles the inosite-fermenting subgroup of *B. coli*—*e.g.* *B. lactis aerogenes*. The colonies exhibit a characteristic mucoid or viscid consistence, already referred to on p. 201.

Biochemical Reactions.—Different strains vary. Some correspond closely with the inosite fermenters of the *B. coli* group (*vide* Table, p. 222*a*). Others are non-lactose fermenters.

This organism may generally be regarded as biologically related to the *B. coli* group, and organisms with similar characters are not infrequently present in faeces.

Occurrence in Human Disease.—Friedländer's bacillus is responsible for only a small percentage of cases of lobar pneumonia. It has been found associated with catarrhal conditions of the respiratory tract, suppuration in nasal sinuses, conjunctivitis, empyema, etc.

BACILLUS OF RHINOSCLEROMA.—This organism closely resembles Friedländer's bacillus in morphology and cultural characters, but produces no gas from glucose, and does not ferment lactose. It is associated with a chronic granuloma of the mucous membrane of the nose, mouth, or throat. The bacilli can be seen in the lesions situated intracellularly. The disease is only prevalent in South-Eastern Europe.

BACILLUS "OZAENAE."—Closely resembles Friedländer's bacillus, but is non-gas-producing in glucose. It is found associated with ozaena, but its aetiological relationship is doubtful.

BACILLUS ACIDOPHILUS AND BACILLUS BIFIDUS

These are Gram-positive bacilli, prevalent normally in the intestinal contents of breast-fed infants, in

which they apparently represent the predominant flora. Their biology and relationships are still somewhat uncertain.

B. ACIDOPHILUS

So called because it is able to flourish in a highly acid medium. In morphology it is a relatively large, non-sporing Gram-positive bacillus, tending to form chains. The individual bacilli vary in length and may even appear in almost coccal form.

It can be cultivated from faeces in bouillon (pH 7.6), to which is added glacial acetic acid, 1 c.c. to 100 c.c. of the medium; it can then be isolated on ordinary agar plates under aerobic conditions (J. Cruickshank).

The *B. acidophilus odontolyticus*, an organism described as the causal agent of dental caries, is apparently a closely related type.

BACILLUS BIFIDUS

Derives its name from the apparently bifid appearance sometimes shown by the organism. It tends to show marked pleomorphism. Though typically Gram-positive, there is a certain amount of variation in its Gram reaction.

In primary culture it is a strict anaerobe, and growths can only be obtained with difficulty.

VIBRIO CHOLERAE

The causative organism of Asiatic Cholera.

Morphology.—Curved or “comma-shaped” rods (vibrios) with rounded or slightly pointed ends, about $1.5\text{--}3\mu$ by 0.5μ . They are actively motile, and the movement is of a “darting” or “scintillating” type. Motility is due to a single long terminal flagellum. The vibrios occur singly, in pairs, or in chains end to end with the curves alternating—*i.e.* presenting a somewhat spiral arrangement. “S” forms and spiral forms representing elongated undivided single cells may be noted. Involution occurs readily, especially in

culture, and globular, club-shaped or irregular forms may be observed. No spores are produced.

Staining.—Gram-negative.

Culture.—Aerobe; slight growth also occurs under anaerobic conditions. Temperature range—12°–40° C., optimum—37·5° C. Grows on ordinary media. A slight trace of acid is inhibitory, and abundant growth occurs on even highly alkaline media.

Colonies on agar—white circular discs about the size of *B. coli* colonies, semi-transparent, with well-defined circular margins; older growths develop a brownish yellow colour.

Gelatin stab—at first there is a white line of growth along the needle track; then liquefaction occurs at the top and spreads downwards in funnel-shaped form.

Potato slope—at first, white layer of growth forms, and later a brownish yellow or pinkish colour is developed; to elicit this growth the reaction of the potato medium must be alkaline.

In bouillon—a uniform turbidity results, and a characteristic pellicle forms on the surface.

Biochemical Reactions.—The fermentative reactions are as follows :—

Glucose	Lactose	Dulcite	Saccharose	Mannite	Maltose
⊥	—	—	⊥	⊥	⊥
	(some strains ⊥ after several days' growth)				

(⊥=acid; no gas)

They can be tested for as in the case of the colityphoid group.

Cholera-red reaction—this depends on the production of indol *and* nitrites in peptone water. It can be elicited by adding a few drops of sulphuric acid to a four-days peptone water culture. A reddish pink colour develops—the “nitroso-indol reaction” due to the interaction of indol, nitrites and sulphuric acid.

Haemolysis—the classical type of *V. cholerae* is non-haemolytic, but haemolytic types have been noted (*e.g.* the “El Tor vibrio”).

Serological Reactions.—From the serological standpoint the cholera vibrio represents a homogeneous species, and unknown strains can be identified by testing their agglutination reaction with an antiserum to a known *V. cholerae*.

Occurrence.—Typical cholera is an acute disease of sudden onset, characterised by intense diarrhoea and tenesmus, vomiting, “rice-water” stools, muscular cramp, and extreme collapse.

The vibrios are present in large numbers in the intestinal contents and dejecta.

Convalescents may remain carriers, and the organism is apparently retained in the gall bladder. The vibrio may remain alive in water for some time, but it cannot exist as a saprophyte.

DIAGNOSIS

The bacteriological diagnosis depends on the isolation and identification of the vibrio.

The organism may be detected microscopically in the intestinal dejecta, but this is not sufficient for accurate laboratory diagnosis, and inexperienced workers may easily be misled by slightly curved bacilli simulating vibrios.

PROCEDURE FOR CULTURE AND ISOLATION

I.—(a) A tube of 10 c.c. peptone water is inoculated with a flake of mucus from the stool, or, in the case of a fluid faecal stool, with a large loopful of the specimen. In examining possible carriers, the stool, if solid or semi-solid, is thoroughly emulsified in sterile salt solution and several loopfuls are added to the medium. The peptone water used is a 1 per cent. peptone with 0.5 per cent. sodium chloride, standardised so as to be neutral to phenol-phthalein—*i.e.* distinctly alkaline to litmus. The tube is incubated for six to eight hours.

(b) A plate of Dieudonné's medium (*vide* p. 73) is also inoculated directly from the stool, and incubated

from twelve to eighteen hours. This medium is highly alkaline and is selective for vibrios, inhibiting the growth of most other intestinal bacteria. A practically pure culture of an intestinal vibrio can be obtained in this way from a stool containing large numbers of *B. coli*.

II. The peptone water culture is examined after six to eight hours, by means of stained films made from a drop of the surface layer of the culture : a large loopful is placed on a slide, and without spreading it, slowly dried at room temperature ; the film is then fixed by heat, and washed in a stream of water to remove the dried peptone particles which stain deeply and obscure the organisms ; the preparation is stained with dilute carbol-fuchsin for one minute and examined microscopically. At the same time a hanging drop preparation is examined ; at the edge of the drop, vibrios are easily detected by their characteristic morphology and "scintillating" or darting motility. In general however, the fuchsin-stained film can be relied on alone for the detection of vibrios. If vibrios are present, a sub-inoculation is made on a Dieudonné plate. If no vibrios are detected, a sub-inoculation is made into a second peptone water tube ; this tube is incubated for six to eight hours, and a film from it is then examined as in the case of the primary culture : if vibrios are present, a Dieudonné plate is inoculated from the peptone culture. If no vibrios are detectable in the second peptone water culture the result may be regarded as negative.

Note.—If vibrios are present in large numbers in the specimen of faeces, an abundant growth can be obtained on the Dieudonné plate inoculated directly from the specimen.

Where vibrios are relatively less numerous, and are not cultured directly on a Dieudonné plate, the organism can be isolated after enrichment in either one or two peptone water cultures.

Pure cultures on agar slopes are obtained from isolated colonies on the Dieudonné plates ; the morpho-

logical, cultural and biochemical characters of the strains are then determined; the final identification of the organism depends on its agglutination by a specific anti-cholera serum—*i.e.* the serum of an animal immunised with a known *V. cholerae* (*vide* p. 137).

Note.—If a direct growth from the stool is obtained on Dieudonné's medium it is, as a rule, practically pure, and the bacteriological diagnosis can be expedited by carrying out the agglutination reaction at once without waiting for the results of cultural tests. Thus within eighteen to twenty-four hours it is possible to determine whether the condition is due to *V. cholerae*. At the same time also it is advisable to obtain cultures from single colonies and confirm the identity of the organism by detailed tests.

THE PARACHOLERA VIBRIOS

These are associated with choleraic conditions, usually of lesser severity than true cholera, and occurring as sporadic cases or in limited outbreaks.

Their morphological, cultural and biochemical characters generally correspond to those of *V. cholerae*, but they are all haemolytic, and liquefy gelatin and solidified serum more rapidly than *V. cholerae*.

They do not react to an agglutinating anti-cholera serum.

These so-called paracholera vibrios represent a number of serological types, differentiated by their agglutination reactions with antisera.

The bacteriological diagnosis in paracholera is carried out in the same way as in true cholera.

BACILLUS PESTIS

The organism of Oriental Plague.

Morphology and Staining.—In its most characteristic form this organism is a short, oval bacillus with rounded ends—*i.e.* cocco-bacillary—about 1.5μ by 0.7μ , occurring singly and in pairs.

It is Gram-negative, and when stained with a weak stain (*e.g.* methylene blue) shows a characteristic

bipolar staining, which is an important feature in identification.

In culture the plague bacillus is less characteristic. Longer forms are frequent, and polar staining is less obvious. Pleomorphism is marked especially in old cultures, and involution or degeneration forms are particularly noticeable. These are markedly swollen, stain faintly, and include globular, pear-shaped, elongated or irregular forms. In fact the microscopic picture of an old culture often suggests that of a yeast or mould. Involution in culture can be hastened by the presence of 3 per cent. sodium chloride, and this is utilised in identifying the organism.

In fluid culture the bacilli tend to be arranged in chains.

The organism is non-motile and non-sporing.

Culture.—Grows aerobically on ordinary culture medium. Unlike other pathogens, the optimum temperature is about 30° C., and it will grow even at temperatures as low as 5° C.

Colonies on agar—small transparent white circular discs, later becoming opaque; they are not specially characteristic.

In older cultures some of the colonies may have outgrown the others and become larger and more opaque. This appearance is not unlike that of a mixed growth.

Gelatin stab—no liquefaction occurs.

Bouillon—growth consists of a granular deposit at the foot and on the sides of the tube, not unlike that of a streptococcus. If grown in a flask of bouillon with drops of sterile oil on the surface, and provided the culture is not subject to shaking or movement, a characteristic growth develops, consisting of “stalactites” hanging down into the fluid from the oil drops.

Biochemical Reactions.—

Glucose	Lactose	Dulcitol	Saccharose	Mannitol
±	-	-	-	±
(±=acid; no gas)				

Animal Inoculation.—The bacillus is pathogenic to monkeys, rats, guinea-pigs and other rodents, and

plague is essentially an epizootic disease among wild rats and certain rodent animals. A guinea-pig or white rat injected subcutaneously with a recently isolated culture dies in a few days, and at autopsy a marked inflammatory condition is noted at the site of injection and in the related lymph glands, with splenic enlargement and general septicaemia. The characteristic bacilli can be seen in large numbers in films from the local lesion, lymph glands, spleen pulp and heart blood, etc. A similar condition is found in rats dying of epizootic plague (*vide infra*).

Rats and guinea-pigs can be successfully inoculated by applying infected material to a shaved area of skin or to a mucous membrane—*e.g.* of the nose.

Occurrence in human lesions.—In *Bubonic Plague* the bacilli are present in large numbers in the affected lymph glands. When the bubo undergoes necrosis as the condition advances, they become less numerous, and may even disappear. Septicaemia may result, and then the bacilli can be detected in the blood during life by blood culture. Post-mortem they are found in the spleen.

In *Pneumonic Plague* the bacilli are present in large numbers in the sputum and in the bronchopneumonic areas in the lung.

In *Septicaemic Plague* the condition is a general septicaemia without definitely localised lesions.

Note.—Plague is epizootic in rats and certain other rodents. The infection is spread by rat fleas (*e.g.* *Xenopsylla cheopis*). The occurrence of bubonic plague in man is due to transmission of the infection from rats by the same agency. The mechanism of transmission is as follows:—The flea sucks blood (containing large numbers of plague bacilli) from an infected animal; the bacilli multiply in the stomach and proventriculus, which may become blocked with bacillary masses: when the insect bites, the contents of the stomach are regurgitated, and so inoculation results.

DIAGNOSIS

Bubonic Plague.—The bubo is punctured with a hypodermic syringe and exudate withdrawn. From

this material, films are made and stained with methylene blue and by Gram's method. The appearance of the characteristic bacilli showing bipolar staining is highly suggestive.

Cultures are also made on nutrient agar, and single colonies are picked off on to agar slopes. The resulting cultures are then available for further investigation.

Some of the exudate should also, if possible, be injected subcutaneously into a guinea-pig or white rat. If plague bacilli are present, the inoculated animal will die, showing at autopsy the appearances, etc., described above.

The cultures obtained should be tested as regards: biochemical reactions, involution on 3 per cent. salt agar, chain formation in bouillon, and stalactite growth. The cultures can also be used for animal inoculation experiments.

Pneumonic Plague.—The bacilli can be detected microscopically in the sputum, and are isolated as in dealing with material from bubonic plague.

In carrying out animal inoculation with sputum, other virulent organisms may be present (*e.g.* pneumococci); instead of injecting subcutaneously, successful inoculation with *B. pestis* can be effected by applying the material to the nasal mucosa, or to a shaved area of skin.

In septicaemic plague, the bacillus can be demonstrated and isolated by blood culture (*vide* p. 131).

DIAGNOSIS OF PLAGUE INFECTION IN WILD RATS

At autopsy the following appearances are noted:—enlargement of lymphatic glands, with periglandular inflammation and oedema, most frequently in the neck glands owing to the fact that the neck is the common harbourage of fleas; serous effusion in the pleural cavity; enlargement of the spleen, which may show small white nodules in the pulp; congestion and a mottled appearance of the liver; haemorrhage under the skin and in the internal organs.

Films are prepared from the heart blood, the glands and spleen, and stained by Gram's method and with methylene blue. Cultures should also be made, and the isolation of the organism attempted by the usual methods. In rats found dead of plague it may be difficult to demonstrate the bacilli microscopically or to isolate them in culture. Carcasses in a state of decomposition may be heavily contaminated with other organisms which render the microscopic examination confusing, and isolation difficult. Inoculation of a white rat or guinea-pig, by smearing the nasal mucous membrane or a shaved area of skin with material from the lesions, should be carried out.

It must be remembered that the plague bacillus is only one species in a biological group (the *Pasteurella* group, including the organisms of "haemorrhagic septicaemia" in various animals) which are similar in morphology, staining, cultural characters and biochemical reactions. One of these, *B. pseudotuberculosis rodentium*, affects rodent animals under natural conditions. This organism is non-pathogenic to white rats, and can be differentiated in this way from *B. pestis*. The haemorrhagic septicaemia organisms can be differentiated by the inhibition or absence of growth on a taurocholate medium (*e.g.* MacConkey's), on which the plague bacillus grows well (Besson).

Attention has also been drawn recently to a plague-like disease of wild rodents due to an organism designated *Bacterium tularensis*. The pathological lesions are not unlike those found in plague-infected animals, and this infection has to be considered therefore, in the diagnosis of plague in animals. The organism is a small rod-shaped structure not usually exceeding 0.7μ in length, and sometimes capsulated. It is present in large numbers in the spleens of infected animals, but it cannot be cultivated like the plague bacillus on ordinary media. Cultures have only been obtained by using a medium consisting of pure egg yolk.

This infection is also transmissible to man—*e.g.* from handling infected animals and from laboratory cultures.

COCCO-BACILLUS MELITENSIS

(MICROCOCCUS MELITENSIS)

The causative organism of Malta or Undulant Fever.

Morphology.—A cocco-bacillus, usually appearing as round or oval forms about 0.4μ in diameter. Definite bacillary forms, however, are frequently observed. The organisms occur singly, in pairs, or even short chains. It is non-motile and non-sporing.

Staining.—Gram-negative.

Culture.—Aerobe. Optimum temperature— 37.5°C . Grows even at 20°C . It can be cultivated on ordinary nutrient media.

Colonies on agar—in primary growth do not appear for three or four days; they are small transparent discs about 1 mm. in diameter, but increase in size to about 3 mm.

Gelatin stab—a delicate line of growth along needle track with a small disc of growth at the top. No liquefaction occurs.

B. melitensis exhibits no fermentative properties.

Occurrence.—The organisms are present in the blood, especially at an early stage. In some cases they may be demonstrated in the urine. Post-mortem they are found in considerable numbers in the spleen and also in various organs. Infection results from the ingestion of the milk of infected goats. Where the disease is endemic (*e.g.* in Malta and the Mediterranean littoral) a considerable percentage of goats are infected, and the organisms can be demonstrated in the milk.

DIAGNOSIS

Blood Culture should be carried out in all cases, and it is essential that 10 c.c. of blood should be withdrawn for this purpose, as the organisms may be relatively scanty.

The agglutination test with patient's serum and a known strain of *B. melitensis* is carried out as a routine procedure. The reaction may be elicited after five days

from the onset of the illness. It has to be noted that normal serum may agglutinate the *B. melitensis* in dilutions even up to 1 in 200, and this introduces a possible fallacy in the application of this diagnostic test. In cases of undulant fever however, the serum often agglutinates *B. melitensis* in high dilutions—*e.g.* 1 in 1000. If, in carrying out the agglutination test in a suspected case, the reaction occurs only with low dilutions (*e.g.* 1 in 100 or 1 in 200), the result cannot be regarded as conclusive. If the test is repeated, a “rising titre” may be observed and a more conclusive result obtained.

In some cases the organism may be isolated from the urine.

Post-mortem it can be cultured from the spleen.

In goats the infection can be recognised by using the agglutination test with the animal's serum and by cultivating the organism from the milk.

THE HAEMOPHILIC GROUP OF BACTERIA

The organisms of this group have very similar morphological characteristics, and are all highly parasitic. They require the addition of blood to the medium for growth to take place. They are extremely delicate, and require frequent subculturing to maintain continued growth.

Members of this group affect man and lower animals; those of medical importance are:

- (1) *B. influenzae* (Pfeiffer).
- (2) *B. pertussis*.
- (3) Koch-Weeks bacillus (*vide* p. 167).
- (4) Ducrey's bacillus (*vide* p. 179).

BACILLUS INFLUENZAE (PFEIFFER)

Originally described as the causative organism of epidemic influenza.

Morphology.—A very small slender bacillus, usually about 1.5μ by 0.3μ , with rounded ends, occurring singly or in pairs; non-motile; non-sporeing. Shorter oval cocco-bacillary forms are also noted, and in culture there is marked pleomorphism.

Staining.—Gram-negative. Does not stain so readily with aniline dyes as other bacteria.

Culture.—Aerobe. Optimum temperature—about 37.5° C. Requires blood or haemoglobin for artificial growth—therefore classified as “haemophilic.” Media containing blood are used for cultivation—*e.g.* blood agar.

On blood agar—very small transparent droplet-like colonies which remain discrete.

It is noteworthy that *B. influenzae* grows better if living in symbiosis with staphylococci, pneumococci, etc.

Experimental Inoculation.—It has not been found possible to produce in laboratory animals a condition corresponding to influenza by experimental inoculation with this organism. The pathogenic effects that have been produced by means of cultures represent, for the most part, only the effects of the endotoxin.

Occurrence.—Found in the sputum, nasal and throat secretions in a considerable proportion of cases of epidemic influenza with inflammatory conditions of the respiratory system. In the inflammatory exudate the bacilli are noted inside leucocytes—*i.e.* intracellular. In the sputum they may occur in exceedingly large numbers, but usually along with pneumococci, streptococci and other organisms associated with inflammation of the respiratory passages. They may be found in the pulmonary lesions in influenzal pneumonia, in empyema and other complications, and have also been noted in the blood in some cases.

It must be remembered that organisms of the type of *B. influenzae* may occur in catarrhal conditions of the respiratory system *apart altogether from epidemic influenza*.

This organism cannot be regarded as the specific primary cause of epidemic influenza, though it may play an important part in the pathogenesis of the pulmonary complications. Its rôle is probably a secondary one.

Haemophilic bacteria similar to *B. influenzae* have also been found in cases of meningitis occurring in young children.

B. PERTUSSIS

Originally described as the causative organism of whooping-cough.

Morphology and Staining.—A very small oval coccobacillus, slightly larger than *B. influenzae*, often showing polar staining; definite bacillary forms are noted, but it is generally more oval in form than *B. influenzae*; non-motile; non-sporing; Gram-negative.

Culture.—Compared with *B. influenzae*, haemoglobin is not so necessary for growth. It is usually cultivated on blood media, but in subculture may grow on serum media without blood pigment. The growth is thicker and more opaque than that of *B. influenzae*, and may be sticky or slimy.

Occurrence.—Present in sputum, especially in the early stages. Nothing conclusive can be stated with regard to the part played by this organism in the aetiology of whooping-cough. It would appear from the evidence available that *B. pertussis* is the causal agent, but this has not been fully proved.

BACILLUS TETANI

The causative organism of tetanus.

Morphology.—Straight, slender, rod-shaped organism about $4-5\mu$ by 0.4μ , with rounded ends; shorter forms and longer filaments are also noted; motile, due to numerous long wavy flagella, peritrichous in arrangement, but the motility is not markedly active. Characteristic spores are developed: spherical, two to four times the diameter of the bacillus, and situated terminally, they produce the "drum-stick" appearance which is a striking morphological feature of the organism.

Staining.—Gram-positive, but different individuals may show variation in the reaction to Gram's stain; by the ordinary methods, only the periphery of the spore is stained.

Culture.—An obligatory anaerobe, but growths can be obtained in the presence of minimal traces of

oxygen; temperature range— 14° – 43° C., optimum— 37.5° C.; grows on ordinary nutrient media; glucose is often added to culture media as a reducing agent; the cooked meat medium described on p. 77 is suitable for the growth of the tetanus bacillus and certain other anaerobes. For methods of anaerobic cultivation see p. 85.

Agar stab culture—no growth occurs on the surface; a white line of growth appears along the needle track, but stopping short of the surface, and lateral spikes develop from the central growth, which are longest in the deeper part of the tube.

Colonies on agar—show a characteristic “feathery” border.

In milk—no coagulation.

Gelatin—is slowly liquefied.

In bouillon—grows well, producing a uniform turbidity.

Cultures have an unpleasant odour, due mainly to sulphuretted hydrogen and methylmercaptan.

The toxin is an exotoxin and can be prepared artificially by growing the organism in bouillon and filtering through a porcelain filter after ten to fourteen days growth. An antitoxic serum can be produced by immunising horses with toxin.

Occurrence.—The spores are practically ubiquitous, but are specially prevalent in manure, and manured soil. Tetanus bacilli may occur naturally in the intestine of certain animals—*e.g.* horses, cattle—and have been noted also in some cases in the human intestinal contents. It is doubtful whether this organism flourishes as a saprophyte in nature, or whether it is derived entirely from an animal source.

Tetanus is usually the result of a wound contaminated with tetanus spores. The infection remains strictly localised, and the tetanic condition is due to the diffusible toxin absorbed into the central nervous system along motor nerve fibres and *via* the blood stream.

Certain conditions favour the propagation of the

organisms in the tissues—*e.g.* sepsis, foreign bodies such as pieces of clothing carried into the wound, *necrotic tissue*, and effused blood.

B. tetani infection may occur in the uterus, as in cases of septic abortion, and also in the umbilical wound of new-born infants.

DIAGNOSIS

- (1) Films may be made from the wound pus or exudate and stained by Gram's method, but the appearance of "drum-stick"-like bacilli is not conclusive evidence of the presence of *B. tetani*, as other organisms occur which are practically identical in morphological characters. Moreover it is often difficult or impossible to detect the tetanus bacilli in wounds by microscopic examination.
- (2) The most reliable method for diagnostic purposes is to produce tetanus in white mice by subcutaneous injection of exudate from the wound, or by the injection of filtrates of anaerobic fluid cultures from the wound.
- (3) Toxin can sometimes be demonstrated in the patient's blood or cerebro-spinal fluid at an early stage of the disease by injecting several cubic centimetres subcutaneously in mice.

THE ANAEROBIC BACILLI OF INFECTED WOUNDS

These organisms are associated with very rapidly spreading inflammatory oedema, necrosis of the tissues and gas production, occurring as a complication of wound infection. They were responsible for the so-called gas-gangrene which was so prevalent in the armies in Europe during the Great War. They are all sporing organisms and their source is mainly animal excreta. The heavily manured soil of the intensively cultivated fields of France contained abundant spores capable of remaining viable for long periods of time. The usual origin of infection is the contamination of wounds with manured soil.

These anaerobes may be divided into two groups: (1) *the saccharolytic*, (2) *the proteolytic*.

(1) The saccharolytic organisms are characterised by their rapid and vigorous growth in carbohydrate media with the production of acid and abundant gas. They have little or no proteolytic activity, so that if grown in a medium containing both carbohydrate and protein—*e.g.* bullock heart medium (*vide* p. 77)—there is rapid production of acid and gas, but *no digestion* of the medium. The cultures have a slightly sour smell, and the meat is turned pink by the acid. Spores are not formed in the presence of carbohydrates.

(2) The proteolytic group are characterised by the digestion of protein. In the bullock heart medium the proteolytic ferments evolved by these organisms break up the muscle fibres with the formation of foul-smelling sulphur compounds, which combine with the iron in the haemoglobin, forming a black compound. This digestion of meat with blackening and foul smell is characteristic of the group. The protein is also broken up into amino-acids, and small white feathery masses of tyrosine crystals are frequently to be seen in cultures. Compared with the saccharolytic group they have very little action on carbohydrates, a small amount of gas only being formed. Their rate of growth is much slower.

The organisms belonging to these groups are the following:—

<i>Saccharolytic</i>	<i>Proteolytic</i>
(1) <i>B. welchii</i>	(1) <i>B. sporogenes</i>
(2) <i>Vibrio septique</i>	(2) <i>B. histolyticus</i>
(3) <i>B. tertius</i> (<i>B. von Hibler IX</i> , or <i>B. Rodella III</i>)	(3) <i>B. putrificus coli</i>
(4) <i>B. fallax</i>	(4) <i>B. tetani</i> (<i>vide</i> p. 236).
(5) <i>B. aerofetidus</i>	
(6) <i>B. oedematiens</i>	
<i>B. botulinus</i> also belongs to this group (<i>vide</i> p. 245)	

There is no hard and fast line of demarcation between the two groups. Thus some strains of *B. welchii* produce small amounts of amino-acids, and *B. aerofetidus* produces sulphuretted hydrogen, but they are both essentially saccharolytic.

The important organisms to be considered here are *B. welchii*, *Vibrio septique* and *B. sporogenes*. *B. tetani* and *B. botulinus* have specific effects which are different from the other members of these groups and are considered separately.

It must be emphasised that the separation and cultivation of these anaerobes is much more difficult than in the case of the aerobes. The ordinary simple laboratory media are ill adapted for anaerobic work, and it is necessary to use media rich in carbohydrate or protein, according to the organism studied. Moreover, the reaction of the medium is of paramount importance, as it is probably only within a certain range or reaction that the bacterial ferments can enable the organism to utilise the food material present.

Having obtained a suitable medium, further difficulties are met with in the separation of the organisms. In wounds there is practically always a mixed infection, so that the isolation of the various bacteria must be undertaken. Simple plating alone is not sufficient as in the case of the aerobes, but alternate growths on plates and fluid media over many generations are necessary before a pure culture is obtained. Much of the chaos in the literature has been due to the failure to separate mixed cultures, and according as the medium contained carbohydrate or protein, so the saccharolytic or proteolytic organisms in the mixtures predominated.

For infection by these anaerobes to take place, *dead tissue or blood clot must be present*. The organisms cannot attack living tissue directly.

During the war, infection occurred where there were deep lacerated wounds caused by irregular-shaped pieces of shell, and into which muddy clothing and particles of earth were carried. Under these

conditions, therefore, spores were introduced into dead tissue under anaerobic conditions and at body temperature.

In the contaminated wound the saccharolytic organisms are the first to grow. Toxic products of growth gain access to the muscle fibre where it is torn, and the toxic fluid causes death of the fibre by passing between it and the sarcolemma sheath. In the dead muscle fibre the saccharolytic organisms (usually *B. welchii*) rapidly multiply, fermenting the muscle sugar and forming abundant gas. The death of the muscle is assisted by the oedema and gas formation, which tend to cut off the blood supply. The process spreads along the injured fibres throughout the length of the muscle. The proteolytic organisms (usually *B. sporogenes*) follow later, digesting the dead muscle and causing blackening and the foul odour.

SACCHAROLYTIC GROUP

BACILLUS WELCHII (*B. PERFRINGENS*, *B. AEROGENES CAPSULATUS*)

The most frequent organism in, and the commonest cause of, gas gangrene.

Morphology.—A relatively large Gram-positive bacillus, about $4-6\mu$ by 1μ , with square or rounded ends, occurring singly or in pairs, and often capsulated when seen in the tissues. In sugar media the bacilli are shorter, while in protein media they tend to become filamentous. The bacilli are non-motile. Spores are formed, but only in the absence of fermentable carbohydrates. They are oval, central, or subterminal, and about the same cross-diameter as that of the bacillus.

Culture.—Anaerobe. Optimum temperature about 37.5°C . Grows best on carbohydrate containing media—*e.g.* glucose agar.

Surface colonies—round smooth regular opaque discs.

In milk—acid, clot and gas production results; the gas breaks up the clot, producing the characteristic “stormy clot” reaction; the culture has a sour, butyric acid odour.

On solidified serum—no liquefaction occurs.

B. welchii is actively saccharolytic and ferments with gas-production, glucose, lactose, saccharose, maltose, starch, and, in the case of some strains, glycerin and inulin.

In the cooked meat medium (*vide* p. 77)—the meat is reddened *and no digestion occurs*.

Exotoxin has been demonstrated in cultures.

Occurrence.—Apart from its pathological relationships, *B. welchii* occurs normally in the intestine. It may invade the blood ante-mortem, and multiplying in internal organs after death, produces the small gas cavities sometimes noted (*e.g.* in the liver) at post-mortem examinations. Apart from wound infections it may occur in uterine infections (*e.g.* septic abortion) and in infections of the intestinal tract and of the urinary system.

Animal Inoculation.—The virulence varies greatly with different cultures. Some strains are markedly pathogenic to guinea-pigs by subcutaneous injection, and the animal may die within twenty-four hours. At autopsy a spreading inflammatory oedema with gas production is noted in the subcutaneous tissue, and necrosis in the underlying muscles, which are sodden, friable and pink.

Pigeons are the most susceptible of laboratory animals to experimental inoculation.

VIBRION SEPTIQUE

Morphology and Staining.—Moderately large bacillus, with rounded ends, about $3-10\mu$ by $0.6-1\mu$. Motile, due to flagella (peritrichous). Tends to grow also in the form of long curved filaments. In the tissues it develops into large, swollen, Gram-positive, lemon-shaped forms, which are designated “citron

bodies." Spores are readily formed and are oval, central, or subterminal, and "bulging." *V. septique* stains Gram-positively as a rule, but degenerate forms are Gram-negative.

Culture.—Anaerobe. Optimum temperature, 37.5° C. Capable of growing on ordinary media.

Agar stab—a white line of growth with short lateral processes.

Surface colonies—irregular transparent droplet-like colonies, later becoming greyish and opaque, with projecting radiations like those of *B. tetani*.

Milk—acid is formed, and the milk is slowly clotted.

Solidified serum—no liquefaction.

Various sugars are fermented.

Exotoxin can be demonstrated in cultures.

Animal Inoculation.—Subcutaneous injection of cultures in laboratory animals produces a spreading inflammatory oedema, with slight gas formation in the tissue. The organisms invade the blood and the animal dies within a day or two. Smears from the liver show long filamentous forms and also "citron bodies."

PROTEOLYTIC GROUP

These organisms (excluding *B. tetani*) are not pathogenic *per se*, but, along with the saccharolytic types, produce intense proteolytic action in the tissues.

BACILLUS SPOROGENES

An anaerobic motile bacillus, with flagella (peritrichous), and oval, usually subterminal spores. It is about the same size as *B. welchii*, but more slender. It is typically Gram-positive in young cultures, but Gram-negative forms are frequent in older cultures.

A stab culture shows a growth like that of *B. tetani*, with lateral radiations or spikes. Surface colonies are yellowish and feathery. Cultures have an exceedingly putrid odour. In milk the casein is precipitated and digested. In cooked meat, the meat is blackened and

digested. Solidified serum is liquefied. A small amount of gas is slowly formed in carbohydrate media.

The organism decomposes protein, producing amino-acids, ammonia, sulphuretted hydrogen, etc.

It is non-pathogenic to laboratory animals.

DIAGNOSIS

The presence of *B. welchii* may be shown by inoculating melted glucose agar in tubes at 50°C. and allowing to solidify. After twelve hours incubation the medium is split up by abundant gas, and the cotton-wool stopper and some of the medium may be forced out of the tube. Microscopical examination shows the typical Gram-positive bacilli. Inoculation into milk, which is incubated under anaerobic conditions, shows the typical "stormy clot" in six to twelve hours.

B. welchii can be isolated in the pure condition by subculturing in a tube of bullock heart medium, and after six hours incubation, sub-inoculating a similar tube. This second culture is plated on agar smeared with alkaline egg medium, and grown anaerobically for twelve hours. Colonies are then transferred to bullock heart medium and two successive inoculations are made at six-hour intervals. Altogether the culture must be passed alternately through two tubes of cooked meat and one plate, nine or ten times, before a pure culture can be hoped for.

B. sporogenes can be detected by inoculating the material into bullock heart medium. After two days incubation, blackening and digestion of the medium with a foul odour is presumptive evidence of the presence of this organism. On microscopic examination, the characteristic subterminal spores or "racquet" forms are seen.

Pure cultures may be obtained by alternate growths for three days in bullock heart medium and three days anaerobic growth on agar plates smeared with alkaline egg medium.

BACILLUS BOTULINUS

The organism of "Botulism," a form of food poisoning characterised by pronounced toxic effects mainly on the parasympathetic nervous system.

Morphology and Staining.—A sporing bacillus with rounded ends, about $4-6\mu$ by $0.9-1.2\mu$, occurring singly and in pairs. Spores are oval, subterminal and slightly "bulging." The bacilli are motile, due to flagella (peritrichous), and stain Gram-positively unless degenerate.

Culture.—Anaerobe. The optimum temperature is about 25°C . Grows on ordinary media, but best on media containing glucose or animal tissue.

Agar culture—a white line of growth, stopping short of the surface, with short lateral spikes or radiations; gas production is marked, especially in glucose agar.

Surface colonies—large greyish, irregular, semi-transparent, often with a central "nucleus." Cultures emit a rancid, butyric acid odour.

Solidified serum—no liquefaction.

Milk—no change.

Ferments glucose actively and to a slight extent various other carbohydrates.

Cooked meat—no blackening or digestion.

In culture medium and in contaminated foods, *B. botulinus* produces a powerful exotoxin which is responsible for the pathogenic effects in the disease.

Two types of the bacillus, designated "A" and "B," have been recognised, producing toxins differing in antigenic specificity.

Occurrence.—Botulism has been found to be due to a considerable variety of preserved foods—*e.g.* ham, sausage, canned meats and vegetables, etc.

In the human subject botulism is not due to the formation of toxin by the organism in the intestine, but to the absorption from the stomach and upper duodenum of *pre-formed toxin produced by the bacillus*.

Canned foods responsible for botulism frequently exhibit distinct signs of spoilage.

B. botulinus is a saprophytic organism and is widely distributed. Its natural habitat is soil, even virgin and forest soil. It may be found on vegetables, fruits, leaves, mouldy hay, ensilage, animal manure.

In cases of botulism the bacillus may be demonstrated in the faeces, and post-mortem in the intestinal contents and in the spleen.

It can also be isolated from the food responsible for the outbreak.

Animal Inoculation.—Laboratory animals are susceptible to experimental inoculation and feeding with cultures. The resulting condition resembles an “acute bulbar paralysis”; at autopsy marked congestion of internal organs, extensive thrombosis and haemorrhages are noted.

Serum antitoxin can be prepared by immunising animals with toxin preparations, and is used therapeutically.

DIAGNOSIS

As the condition of botulism is essentially a food intoxication, the suspected food calls for investigation.

It is macerated in sterile salt solution, heated at 60° C. for half-an-hour to eliminate non-sporing bacteria, and then cultures are made under anaerobic conditions at 25° C. In this way *B. botulinus* can be isolated and identified.

An extract should also be made from the food, sterilised by filtration through a porcelain filter, and injected subcutaneously into a guinea-pig.

Cultures isolated from the food should also be investigated by animal experiment.

ACTINOMYCES

The causative organism of actinomycosis in animals and man.

This organism belongs to the genus *Streptothrix* of

the Higher Bacteria (*vide* p. 15), and may be more correctly designated "Streptothrix actinomyces."

Morphology.—The organism grows as a mycelium or felted mass of branching filaments which are comparatively slender (0.6μ thick) and are enclosed in a sheath containing pigment granules. In the centre of this mycelial colony the filaments interlace irregularly, but at the periphery there is a tendency to radial arrangement. The organisms show true dichotomous branching. The mycelium is embedded in a ground-work or matrix. In old growths the filaments become matted together into a structureless mass. They also show fragmentation into bacillary and coccial forms. Some divide up into rows of oval or spherical spore-like structures or gonidia. Growing in the tissues (especially in animals) the actinomyces colony develops pyriform or club-shaped structures at the periphery, resulting from the swelling of the sheath at the extremities of peripheral radial filaments. These "clubs" lie radially with their wide end outwards and form a complete ring round the colony. In animal lesions the clubs may constitute the only morphological feature of the older colonies, owing to the degeneration of the filaments, which become fused into a structureless mass in the centre of the colony. The clubs are regarded as a defensive mechanism on the part of the organism against the tissues of the host. In human lesions, club formation is comparatively rare.

Staining.—The filaments are Gram-positive. The clubs usually stain Gram-negatively, but are acid-fast, and can be differentially stained by the Ziehl-Neelsen method, substituting 1 per cent. for 20 per cent. sulphuric acid.

Culture.—Two main cultural types are recognised :

- (1) Boström's type, which is aerobic, and
- (2) The anaerobic type of Israel and Wolff, which is the more frequent in animal and human lesions.

Boström's Aerobic Type.—Temperature range— 20° – 40° C., optimum— 37° – 38° C. Grows on ordinary

media, but the presence of serum or glycerin encourages growth.

On agar—grows slowly; colonies begin to appear after four or five days; when well developed, they stand out on the surface of the medium as discrete, rounded, yellow, transparent knobs, often likened to “amber drops”; they are firmly adherent to the medium; older colonies become umbilicated and assume a dry appearance.

In gelatin—slow liquefaction occurs.

Anaerobic Type.—The optimum temperature is 37°–38° C. and growth does not occur at temperatures much below the optimum.

On agar—colonies are raised, greyish white, opaque discs which tend to show a rosette form and are firmly adherent to the medium.

A shake culture in a tube of agar shows a characteristic distribution of the colonies, which are most numerous in a zone from 10–20 mm. below the surface—*i.e.* where there is still a trace of free oxygen present.

If the organism is grown in the form of a stab culture in agar, growth is also at an optimum in a similar zone. Though for all practical purposes this organism is anaerobic, a minute trace of oxygen is more favourable to growth than complete anaerobiosis.

Occurrence.—Actinomycosis is an infective granuloma, occurring mainly in cattle, sheep and pigs, and occasionally in man. In human cases the lesions usually show a suppurative tendency, and the pus contains colonies of the parasite in the form of small round granules about the size of a pin head, which are sometimes of a bright yellow colour (like grains of sulphur). These granules can be recognised by the naked eye if the pus is examined in the form of a thin layer on a slide. The commonest avenue of infection in man is through the mucosa of the mouth or throat. The mode of infection is often doubtful, and it may be impossible to trace the source. Primary foci have been noted in man and animals, around fragments of grain embedded in the mucous membrane of the mouth. It

has been thought that grain might be a primary source of the infection, but it is more likely in these cases that the parasite has gained independent access to the body, and that the grain fragment facilitates the establishment of the organism in the tissue. Where the avenue of infection is by the mouth or throat, the primary lesions involve the soft tissues of the mouth and neck, the periosteum of the jaw, and even the vertebrae. In some cases the avenue of infection may be through the mucosa of the bowel (*e.g.* caecum), or the primary lesion may be in the lung.

Metastatic lesions are also liable to occur (*e.g.* in the liver, brain, kidneys, or lung) by blood stream spread.

The organism is found both in pus and in tissue lesions in the form of compact colonies or granules which are visible even to the naked eye, and these present the microscopic appearances described above, varying according to the age of the individual colonies.

BACILLUS ACTINOMYCETUM COMITANS. — Besides the mycelial organism, a small Gram-negative coccobacillus is frequently present in large numbers in the actinomyces colony. This organism can be cultivated independently. Growth occurs under aerobic conditions, and consists of small moist semi-transparent colonies entirely different from those of the streptothrix. This organism has been regarded as a distinct species, and has been designated *B. actinomycetum comitans*, but its actual relationship to the actinomyces is uncertain.

DIAGNOSIS

If the pus from an actinomycotic lesion is spread out in a thin layer in a Petri dish or on a microscope slide, the characteristic colonies or granules can be recognised with the naked eye. For microscopic examination the granules in a drop of pus are "crushed" between two slides. In this way films can be prepared and then stained by Gram's method.

The granules can easily be separated by shaking up the pus with water in a test-tube, allowing them to sediment, and collecting them in a capillary pipette. They are then deposited on a slide and films made by crushing. Preparations obtained in this way are more satisfactory than those made directly from pus in which the granules may be relatively scanty.

Microscopic examination is generally sufficient for clinical diagnosis—*i.e.* the presence of Gram-positive filaments arranged in the form of a mycelium.

In tissue lesions the colonies can be recognised by preparing histological sections and staining by Gram's method, and, in the case of animal lesions, by the modified Ziehl-Neelsen method described above.

To cultivate the organism it is essential that actual granules should be used for inoculating the medium. For this purpose, the pus is mixed with sterile water, the granules are allowed to sediment, and then removed with a pipette; this is repeated two or three times so that the granules are thoroughly washed. This procedure is particularly necessary where the pus represents a mixed infection. Two agar plates are inoculated with the separated granules. One is incubated aerobically, the other anaerobically.

OTHER PATHOGENIC STREPTOTHRICES

Apart from actinomycosis, granulomatous and suppurative conditions occur in animals and man, due to infection with streptothrices, which differ biologically from the actinomyces. It is unnecessary to refer to these in detail. The so-called Eppinger's streptothrix and the *Streptothrix madurae* of *Mycetoma* may be taken as examples.

EPPINGER'S STREPTOTHRIX (*S. ASTEROIDES*)

Originally isolated from a brain abscess. The filaments are relatively broad (about 2μ in diameter), and very readily break up in culture into bacillary forms. They stain Gram-positively. This organism

can be cultivated readily on ordinary medium as a friable, white, dry, wrinkled or nodular growth, which later becomes pigmented (yellow or pink).

THE ORGANISMS OF MYCETOMA OR MADURA FOOT

Mycetoma is an infective granuloma localised usually to the tissues of the foot, and exhibiting no metastases. The condition only occurs in certain tropical and subtropical countries (*e.g.* India, some parts of Africa, etc.).

In the tissue lesion and pus, granules or colonies are noted as in the case of actinomycosis. These granules vary in colour; in some cases they are white or yellow ("pale variety"), in others black ("melanoid variety").

The *pale granules* usually represent colonies of a streptothrix—the *Streptothrix madurae*.

Morphologically this organism resembles the actinomyces, but clubs are less frequently noted.

Cultural characters.—It is a strict aerobe. The optimum temperature is about 37·5°C. On nutrient agar the growth consists of circular raised colonies like those of the actinomyces, at first yellowish, later pinkish.

The *black granules* in mycetoma represent a hyphomycete, which has been designated *Madurella mycetomi*.

The colonies consist of a mycelium of broad, branching, septate hyphae 3–8 μ in breadth, and contain a considerable amount of black pigment.

This organism can be cultured on nutrient agar, and old cultures show the black pigmentation.

LEPTOTHRIX (*vide* p. 14)

An organism of this type is a common inhabitant of the mouth cavity, and may be detected in films made from the secretion or deposits of tartar between the teeth. It is designated *Leptothrix buccalis*. Pathogenic properties have been claimed for it, but this is doubtful.

Leptothrix types have also been reported in suppurative lesions in the region of the mouth and throat.

THE HUMAN MYCOSES

Hyphomycete and blastomycete infections (*vide* pp. 13, 14) are usually designated "mycoses."

The more important of these are due to organisms of the group of so-called **FUNGI IMPERFECTI**—*i.e.* fungi which possess no characteristic fruiting organs, and are difficult to classify with the more distinctive groups. This group includes Microsporon, Trichophyton, Achorion, etc.

MICROSPORON

This organism is the common cause of scalp ringworm.

The mycelium develops in the scalp epidermis, and in the hair medulla from which hyphae pass through the cortex, and produce a covering of small spherical spores (approximately 2μ in diameter) arranged in the form of a mosaic on the outside of the hair. Grown artificially, microsporon develops large circular colonies with a raised centre and radiating folds. The surface of the colonies is velvety, due to the projecting aerial hyphae. Pigmentation is often marked, and the actual colonies become brown in colour though the surface layer remains white or buff-coloured.

Various species have been recognised — *e.g.* *M. Audouini*, *M. canis*.

TRICHOPHYTON

This organism is the characteristic parasite of ringworm affecting the bearded area. It may also occur in ringworm of the scalp, nails and glabrous skin.

The fungus mycelium develops in the hair medulla as a rule, and produces characteristic chains or "rosaries" of oval or rectangular spores, which are larger ($3-5\mu$ or even 8μ) than those of microsporon. In artificial culture the different species vary in

detailed characters, but present the same general type of growth as the microsporon. A common species in this country occurring in scalp ringworm is *T. crateriforme*, which produces raised colonies with crater-like depressions. Some species are markedly pigmented—e.g. *T. rosaceum*, *T. violaceum*.

ACHORION SCHÖNLEINII

Associated with the condition called “favus,” which affects the scalp and other skin areas.

The fungus forms the characteristic concave yellow discs or “scutula” centred round hair follicles. The scutulum is composed of mycelium and spores, which are markedly irregular in size and arrangement. The hairs in the affected area are also invaded. The artificial growth on culture medium consists of a tough brownish layer with raised irregular folds, and a white velvety surface due to the aerial hyphae.

Microsporon furfur of pityriasis versicolor, *Endodermophyton concentricum* of tinea imbricata (in which the mycelial growth occurs between the rete Malpighi and stratum corneum of the epidermis), *Endodermophyton Castellani* of tinea intersecta, and *Epidermophyton cruris* of “dhobie itch,” are further examples of “fungi imperfecti” responsible for human dermatomycoses.

Sporotrichon.—Associated with subcutaneous ulcerating granulomata. The condition is designated “sporotrichosis.” When grown artificially, the organism forms a typical mycelium; the hyphae are relatively narrow (2μ); oval spores are found in clusters at the ends of hyphae. In the tissues it may be difficult to demonstrate the organism by microscopic examination; the morphology is entirely different, and no definite mycelium is observed; oval or spindle-shaped forms are found; some of these are not unlike large bacilli; budding may be noted as in the case of the blastomycetes.

Madurella, a hyphomycete found in mycetoma, is referred to on p. 251.

PHYCOMYCETES

Mycelium made up of non-septate hyphae ; asexual spores developed in a spherical "sporangium" or spore-case borne on the expanded end of an aerial hypha ; "zygospores" also formed as the result of conjugation of two hyphae at their tips. These fungi are of little or no importance as pathogens, though they have been noted in auricular, naso-pharyngeal and pulmonary mycoses, and in mycosis of the tongue. They are frequent contaminants of food, and of culture medium in the laboratory. The common mould *Mucor mucedo* exemplifies the group.

ASCOMYCETES

Mycelium typically septate ; "ascospores" developed in a spore case ("ascus") formed at the end of a hypha. The moulds *Aspergillus* and *Penicillium* are examples of this group.

Aspergillus is a frequent contaminant of culture medium, occurring as a felted yellow, green, or black, mould growth ; rows of spores or "conidia" develop from finger-like processes or "sterigmata" borne on an expanded aerial hypha. Pulmonary aspergillosis may occur in birds—*e.g.* pigeons—and this infection has also been noted in man (among bird fanciers). *A. pictor* is responsible for a skin disease of Central America called Pinta, but other species of fungi are also associated with different varieties of this condition.

Penicillium glaucum is one of the commonest food moulds and contaminants of culture medium. Its biology is similar in many respects to that of *aspergillus*. Cultures assume a characteristic dull green colour.

BLASTOMYCETES

(SACCHAROMYCETES, YEASTS)

These are often classified with the Ascomycetes, and the yeasts are now regarded more as a phase of other types of fungi than as a separate group. Their

morphology, and gemmation or budding, are highly characteristic features, and though in some species elongation into hyphae occurs, it seems justifiable to consider them separately from the hyphomycetes or moulds.

Pathogenic types :

Cryptococcus Gilchristi—of Blastomycetic dermatitis.

Cryptococcus linguae pilosae—of “Black-tongue.”

Coccidioides immitis—of “Blastomycosis” (an uncommon granulomatous or suppurative condition involving skin, subcutaneous tissue, lungs, etc., or a generalised pyaemic condition).

(*Cryptococcus farciminosus*—of Epizootic Lymphangitis in horses.)

Monilia.—This type is yeast-like in morphology and exhibits the characteristic gemmation, but the cells tend to elongate into filaments.

M. candida is the species found in thrush. This organism possesses active fermentative properties, and produces in culture, acid and gas from various carbohydrates.

M. psilosis has been described as the causative organism of Sprue, but its aetiological relationship is uncertain.

DIAGNOSIS OF THE DERMATOMYCOSES

The affected hairs, or the epithelial scales from affected areas, are treated on a microscope slide with a 10 per cent. solution of caustic potash, gently warmed, and examined in the unstained condition under a cover-glass with a $\frac{1}{8}$ -in. lens. The alkali clears the hairs and epidermis, and renders the fungi easily detectable under the microscope. Stained preparations are unnecessary for routine diagnostic work.

For artificial culture, Sabouraud's medium, standardised + 20 by Eyre's method, is the most suitable medium, and it tends to restrain the growth of bacteria. A layer of the medium is solidified in the bottom of a conical flask. In making cultures from scales and hairs, two minutes immersion in absolute alcohol before “planting” them on the medium assists in the isolation of the fungus by destroying the cocci and other bacteria that may be present on the skin.

The mouth of the flask is covered with a rubber cap over the cotton-wool plug, and the cultures are incubated at 25° C. The growth of the fungus may be relatively slow, and the cultures may have to be incubated over a prolonged period to elicit characteristic appearances.

THE SPIROCHAETES OF IMPORTANCE IN MEDICINE

SPIRONEMA PALLIDUM

(SPIROCHAETE PALLIDA, TREPONEMA PALLIDUM)

The causative organism of syphilis.

Morphology.—An exceedingly delicate, spiral filament 6–15 μ by 0.25 μ , with six to twelve coils which are comparatively small, sharp, and regular. The ends are pointed, and at each end a flagellum has been demonstrated. The organism is feebly refractile, and can only be demonstrated in the unstained condition by dark-ground illumination (*vide* p. 42).

The organisms show a rotatory corkscrew motility, and also movements of flexion.

Staining.—*Spirochoma pallidum* is difficult to stain, and cannot be demonstrated by the ordinary staining methods. It can be stained by Giemsa's solution applied in a 1 in 10 dilution over a prolonged period (twenty-four hours), and appears faint pink in colour. For the demonstration of this organism in films, Fontana's silver impregnation method (*vide* p. 115) is the best available in routine work. In tissues, the spironemata are well demonstrated by Levaditi's silver impregnation method (p. 116).

Culture.—Has been grown anaerobically by Noguchi. The technique of isolating pure cultures is as follows:—Deep tubes of 2 parts agar and 1 part ascitic fluid are prepared, with a piece of sterile tissue added (*e.g.* rabbit kidney). The medium is covered with a layer of sterile vaseline. The tubes are inoculated from exudate with a capillary pipette

(stab inoculation). Both the spironemata and the bacteria present in the material grow along the line of inoculation, but later, the spirochaetes spread out from the stab and grow in the form of a haze in the medium. The tube is cut, and transplants are made in Smith-Noguchi medium (*vide* p. 73), without carrying over any of the other bacterial growth. In this way pure cultures are obtained.

Occurrence.—In the primary stage, spironemata are present in large numbers in the chancre and in the exudate from it, but as the sore tends to heal they become less numerous, and may not be demonstrable in the exudate. They are also present in the buboes.

In the secondary stage, spironemata have invaded the blood stream and become widely distributed in the body. They are present in the roseolar rash, mucous patches, condylomata, and may even be observed in the blood.

In the tertiary stage, they are less easily demonstrated in lesions, but can be observed by suitable staining methods in the periphery of gummata, in the arterial lesions, etc.

In general paralysis of the insane spironema pallidum has been demonstrated in the cerebral cortex.

In congenital syphilis, spironemata are found in certain internal organs—*e.g.* liver—often in very large numbers. They are present also in the skin lesions, the blood, and the mucosa of the intestine and bladder.

SEROLOGICAL REACTIONS

Wassermann Reaction.—Reference has been made to this manifestation of syphilis on pp. 138–145, and the technique has been described.

The reaction appears in about two to four weeks after the onset of the primary lesion, but its development may sometimes be delayed. In secondary syphilis the reaction is usually well marked and fairly constantly present (95 per cent. of untreated cases).

It is less frequently positive in the latent stage (25–50 per cent. of cases) and in tertiary cases (75 per cent.). In general paralysis and in many cases of locomotor ataxia, the reaction is positive both when tested with blood and with cerebro-spinal fluid. In cerebro-spinal syphilis the spinal fluid may react positively even when the blood yields a negative reaction. Active cases of congenital syphilis usually exhibit a strongly positive reaction.

As the result of antisyphilitic treatment the Wassermann reaction may become negative, but often temporarily, and the reaction reappears when treatment is stopped.

Positive reactions have been recorded in leprosy and malaria, but in assessing the significance of these results, it must be remembered that concomitant syphilis may be responsible for Wassermann reactions noted in other diseases. The occurrence of positive reactions as a result of leprosy and malaria is subject to considerable doubt. The Wassermann reaction may be positive in other spirochaetal diseases—*e.g.* Yaws, Relapsing Fever, and also in Trypanosomiasis.

The Sachs-Georgi Reaction is dealt with on p. 145. It follows very closely the Wassermann reaction in its occurrence, and corresponds quantitatively with the complement-deviation reaction.

DIAGNOSIS

In the primary stage, when there is an ulcerated sore, *Sp. pallidum* can usually be demonstrated in the serous exudate from the ulcer. The dark-ground illumination method is the ideal technique for the purpose, and provides a convenient means of rapid diagnosis. Failing this, Fontana's staining method can be used.

Sp. pallidum is recognised by its special morphological features, and must be carefully differentiated from other spirochaetes found in ulcerating sores—*e.g.* *Sp. refringens*, *Sp. gracilis* (*vide infra*).

Directions for obtaining a specimen of exudate from a syphilitic sore for microscopic examination:

The serous exudate should be obtained from below the surface, and should not include surface organisms, as other spirochaetes which may be confused with the *Sp. pallidum* are frequently present. The presence of excessive numbers of red blood corpuscles in the specimen is also to be avoided, as they tend to obscure the spirochaetes. When a case is submitted for microscopic diagnosis it may be found that a local antiseptic has been applied; in such cases it may not be possible to find the spironema until a wet dressing of gauze soaked in sterile saline solution has been applied to the sore for twenty-four to forty-eight hours. It is to be noted also that antisymphilitic treatment, initiated before the examination, diminishes the likelihood of successful microscopic diagnosis.

The sore is cleansed with a swab soaked in warm saline solution, and the margin is then scraped lightly with some blunt instrument to abrade the superficial epithelium. On squeezing the base of the chancre, serum exudes, and if blood-stained, should be removed with dry gauze until clear exudate can be obtained. Some of this is then collected in one or two capillary tubes. Both ends of the tubes are sealed in the flame, and the specimens are submitted for examination.

When the primary sore is in process of healing, microscopic examination of the exudate may yield negative results. At this stage spironemata may be found in the fluid aspirated from the buboes by means of a syringe.

In the secondary stage spironemata may also be demonstrated in the serum from the skin eruption, and in the exudate from mucous patches, etc. Serum can be obtained from the skin eruption by scarifying and "cupping" with a test-tube.

After about two weeks from the onset of the primary sore, the Wassermann reaction can be employed for

diagnosis. The reaction becomes progressively more pronounced with the advance of the disease, and is usually markedly positive in the secondary stage.

Owing to the fact that the reaction may be slow in developing, if at first a negative result is elicited in the primary stage, it is essential to repeat the test before excluding syphilis. A negative reaction in a suspected case of secondary syphilis is highly significant in excluding syphilitic infection, but in supposed latent or tertiary cases a negative result does not exclude the disease.

In cerebro-spinal syphilis both the blood and spinal fluid should be tested.

In dealing with cases of congenital syphilis in young infants, the mother's blood should be tested if there is any difficulty in obtaining a specimen of blood from the child.

It may be said that the Wassermann reaction, carried out by a reliable technique, is clinically specific for syphilis among the diseases of a temperate climate.

The Sachs-Georgi reaction may be substituted for the Wassermann test, and is simpler to carry out, but at the present time most laboratory workers prefer to rely on the complement-deviation reaction.

SPIRONEMA PERTENUE

The causative organism of Framboesia or Yaws, a tropical disease pathologically and clinically resembling syphilis.

Morphology.—Practically identical with the *Sp. pallidum*. When first described it was regarded as more delicate than *Sp. pallidum*—hence the designation.

Its occurrence in lesions corresponds to that of the *Sp. pallidum*, and the diagnosis of infections is carried out as in syphilis. The Wassermann reaction is positive.

SPIRONEMATA OF THE "REFRINGENS" TYPE

These are large, motile, refractile spirochaetes, with irregular wide and open coils which are relatively few

in number. They are easily stained by the ordinary methods. They occur in various gangrenous and ulcerative conditions on the surface of the body, the mouth and throat, and the genitals. This type of organism may be found in the surface exudate of a syphilitic sore, and has to be differentiated morphologically from the *Sp. pallidum*.

"*Sp. vincenti*" is of this type, and occurs in a pseudo-membranous condition of the throat—Vincent's angina. It is usually associated with a large fusiform bacillus—*B. fusiformis* (*vide* p. 266). Films from the throat secretion, stained by Gram's method or dilute carbol-fuchsin, show large numbers of spirochaetes.

"*Sp. balanitidis*" is associated with balanitis gangrenosa. Biologically it is of the refringens type, and is usually associated with *B. fusiformis*.

"*Sp. buccalis*" is a similar type, so called owing to its occurrence in the mouth—*e.g.* in ulcerative gingivitis.

SPIRONEMA GRACILE

This organism may occur in the secretions of the genitals, and morphologically resembles *Sp. pallidum*. Its differentiation from the latter is therefore of practical importance in syphilis diagnosis. It is not usually found if care has been taken to obtain serum from below the surface of the chancre (*vide supra*). It is thicker than *Sp. pallidum*, and by the dark-ground-illumination method appears "glistening," whereas *Sp. pallidum* is "dead white."

SPIRONEMA MICRODENTUM

This organism flourishes in carious teeth, and may be found in the secretion between the teeth. It closely resembles *Sp. pallidum* in morphology, but it is shorter ($4-10\mu$), and the coils are closer together and not so deep. It is more easily stained by the ordinary methods than *Sp. pallidum*.

THE SPIRONEMATA OF RELAPSING FEVER

SPIRONEMA OBERMEIERI

The causative organism of European relapsing fever.

Morphology and Staining.—This organism is a spiral filament, varying in length as a rule, from 15–30 μ , and about 0.5 μ broad, with six to fifteen fairly regular coils. Active motility of a rotatory or oscillating type is noted in fresh preparations. Single terminal flagella have been observed. Multiplication is by longitudinal and transverse fission.

This spirochaete stains readily with a Romanowsky stain, and may exhibit uniform staining or beading. It can be stained also with carbol-fuchsin, and is Gram-negative.

Culture.—Artificial cultures can be obtained in Smith-Noguchi medium. Citrated blood containing spironemata from an infected animal—e.g. a white rat—is used as the inoculum.

Occurrence.—This organism is present in the peripheral blood during the pyrexial stage of the illness, and can easily be detected in blood films. When defervescence occurs it disappears from the blood, but may still be present in considerable numbers in the spleen, where it is phagocytosed by large mononuclear cells.

It is transmitted from person to person by the body louse, *Pediculus vestimentorum*. After this insect has sucked blood from an infected individual, multiplication of the organisms occurs in the stomach, and possibly the spirochaete goes through a phase in its life-history in the insect's body. Infection results either through the contamination of the bite wound with the infective excreta of the louse, or by the crushing of the infective lice with the fingers in the act of scratching, and by the simultaneous inoculation of the abrasions.

Animal Inoculation.—Monkeys, white mice and

white rats can be experimentally infected by subcutaneous injection of blood from a case of relapsing fever.

SPIRONEMA DUTTONI

The organism of West African relapsing fever (African Tick Fever).

This organism is biologically similar to *Sp. Obermeieri*, but probably represents a separate variety or species. It has been described as longer than *Sp. Obermeieri* and with less regular coils. Its distribution in the disease is also similar, but it is transmitted by a tick (*Ornithodoros moubata*). In the tick, it apparently goes through some stage in its life-cycle, and may be passed to the second generation from an infected female insect. "Chromatin" granules have also been observed in the spirochaete. These are extruded, and have been regarded as a phase in the life-history of the organism. These granules have been noted in the Malpighian tubules of infective ticks.

The *Spirochaetes* found in *American, Asiatic and East African relapsing fevers* possibly represent separate varieties or species.

DIAGNOSIS IN RELAPSING FEVER

During the pyrexial phases, the spirochaetes can easily be demonstrated in the blood, but not during the apyrexial intervals.

Thin blood films are made as in malaria diagnosis, and stained by Leishman's method (*vide* pp. 112, 270).

Some workers prefer to stain the films with carbolfuchsin.

If a drop of blood is mounted on a slide under a cover-glass and examined with the oil-immersion lens, the spirochaetes show active movement, and can be detected in the unstained condition.

SPIRONEMA MORSUS MURIUM

A spirochaete found in cases of rat-bite fever.

It has been described as a short spirochaete $2-5\mu$

long, and relatively broad, with a few regular curves and two long terminal flagella. It is found locally in the inflammatory lesion at the site of the bite, and in the lymph glands and blood. Motility is exceedingly active.

It can be stained readily by the ordinary aniline dyes.

Successful cultures have been obtained in Smith-Noguchi medium.

The organism is pathogenic to white rats, mice, guinea-pigs.

Apparently this spirochaete occurs naturally in wild rats, as in the case of the *L. icterohaemorrhagiae* (*q.v.*).

LEPTOSPIRA ICTERHAEMORRHAGIAE

The causative organism of Infectious Jaundice (Weil's Disease).

Morphology and Staining.—A leptospira (*vide* p. 19) about $6-9\mu$ long (average) by 0.25μ broad. The coils are very numerous and so fine that they are difficult to demonstrate in stained preparations, though quite obvious by dark-ground illumination. Hooked ends are a characteristic morphological feature. Active movement is observed in fresh preparations examined with the dark-ground microscope. The movement is both rotatory and undulating.

The organisms can be demonstrated by Giemsa's stain (as in the case of *Sp. pallidum*) and by the silver impregnation methods of Levaditi and Fontana.

Culture.—*L. icterohaemorrhagiae* can be cultivated readily in Noguchi's leptospira media (*vide* p. 76), and grow just below the surface. The optimum temperature is from 25° – 30° C.

Occurrence.—The organisms are present in the blood during the first six days of the illness, and though scanty, can in some cases be demonstrated microscopically in blood films. Later they disappear from the blood. They are present in the liver often in

considerable numbers, and particularly in the later stages of the disease, in the kidneys, when they can be detected in the urinary sediment.

Leptospira icterohaemorrhagiae occurs in wild rats, which act as carriers of the infection. In these animals the spirochaetes are present in the kidneys, and are excreted in the urine. In this way soil, food, etc., are contaminated. Infection of the human subject may occur by the alimentary tract, but it has been shown also that the organisms can pass through skin that has become sodden by continuous wetting. In Japan, epidemics of Infectious Jaundice have been specially noted among workers in wet mines. Similarly during the Great War outbreaks occurred among troops in wet trenches. The contamination of soil by the urine of rats harbouring the leptospira is undoubtedly an important factor in the transmission of the disease, and invasion occurs through skin abrasions or the intact wet skin—*e.g.* in the case of the Japanese miners working barefooted.

DIAGNOSIS

During the first six days of the disease 5 c.c. of blood are withdrawn by vein puncture and injected intraperitoneally into a guinea-pig. The animal dies in eight to twelve days, showing a marked jaundice with haemorrhages in the lungs, under the serous membranes and in the muscles. The leptospira is present in large numbers in the liver and kidneys, and can also be found in various other organs and in the blood.

The methods of demonstration in the stained and unstained conditions have been referred to above.

If a case is met with only at a later stage—*e.g.* ten to fourteen days from the onset—the urine is centrifugalised and the sediment examined by the dark-ground microscope. A guinea-pig should also be inoculated intraperitoneally with the centrifuged deposit.

LEPTOSPIRA ICTEROIDES

Described by Noguchi as the causative organism of Yellow Fever.

By injecting blood from cases of Yellow Fever into guinea-pigs, Noguchi found that in a certain proportion of the experimental animals, a pathological condition developed which corresponded to that of the human disease, and in the blood, liver and kidneys of the infected animals, a leptospira was observed morphologically similar to *L. icterohaemorrhagiae*.

The organism was cultivated both from the experimental animals in *Noguchi leptospira media* (*vide* p. 76), and also in a few human cases from the blood; the cultures reproduced the disease in guinea-pigs.

The leptospira was demonstrated with the dark-ground microscope in the blood of a small proportion of the cases investigated.

It is to be noted that the transmission of the virus of Yellow Fever by mosquitoes (*Stegomyia fasciata*) had been established by previous investigators, and the virus was regarded as a filter passer.

BACILLUS FUSIFORMIS

This organism has been referred to as a concomitant of spirochaetes of the refringens type, and is found in various necrotic inflammatory conditions along with these spirochaetes—*e.g.* in Vincent's angina, ulcerative stomatitis, etc.—and occasionally in diphtheritic lesions of the throat.

Morphology.—It is a large, non-motile, fusiform bacillus, about 10–14 μ by 1 μ .

Staining.—Gram-negative. The centre of the bacillus often stains less deeply than the poles, and a beaded or granular appearance may be noted.

Culture.—Anaerobe. It has been cultivated on a medium containing 1 part of blood to 3 of agar. The colonies are small white discs resembling a growth of streptococci.

THE MICRO-ORGANISMS OF MALARIA

Malaria is a protozoal disease in which the causative organism—*Plasmodium*—invades the red cells of the blood. Three species of the malaria plasmodium are recognised:

P. vivax—Benign tertian malaria.

P. malariae—Quartan malaria.

P. falciparum—Malignant malaria.

These organisms belong to the order Haemosporidia of the Sporozoa. The plasmodium is transmitted by anopheline mosquitoes, and goes through the sexual phase of its life-cycle in the body of the insect.

Outline of the life-history of the Malaria Plasmodium.—It is introduced into the body by the bite of the mosquito (*vide infra*) as a minute spindle-shaped cell or sporozoite, containing nuclear material in the form of a granule of chromatin. The sporozoite enters a red cell, and becomes an amoeboid structure (amoebula), which gradually develops in the cell (trophozoite). It displays amoeboid movement and protrudes pseudopodia. It grows at the expense of the red cell and accumulates altered blood pigment in the form of brownish granules. More than one trophozoite may attack a single corpuscle. The corpuscle may show considerable alteration in size as the trophozoite develops. The trophozoite tends to assume a characteristic ring form, with the chromatin granule or “dot” at one side, so that the whole structure resembles a signet-ring. When full-grown it is more or less rounded, and may occupy the greater part of the corpuscle. These “mature trophozoites” usually contain a considerable amount of blood pigment.

When fully developed, the trophozoite undergoes schizogony. The chromatin breaks up into smaller particles, the protoplasm subdivides and forms a number of small round or oval merozoites (about 2μ in diameter) each containing a fragment of the original chromatin. The residual protoplasm and pigment remain in the centre of the group of merozoites. The

number of merozoites resulting from schizogony varies with the different species (*vide infra*). Finally the corpuscular envelope breaks, and the individual merozoites are liberated as free structures in the blood. The merozoite invades another red cell and thus the asexual cycle is repeated. The pigment is taken up by leucocytes.

The length of time the organism takes to complete the asexual cycle varies with the species :

<i>P. vivax</i>	Two days
<i>P. malariae</i>	Three days
<i>P. falciparum</i>	One or two days.

The fever develops at the stage of schizogony ; thus in *P. vivax* infections the febrile paroxysm occurs every third day (tertian malaria), in *P. malariae* infections every fourth day (quartan malaria).

In malignant malaria, schizogony only occurs as a rule in the blood of internal organs, and not in the peripheral blood.

While some of the organisms develop into schizonts, others become gametocytes. In the case of *P. vivax* and *P. malariae*, these are rounded and about the same size as a mature trophozoite. The gametocytes of *P. falciparum* are sausage-shaped or crescentic, with the envelope of the corpuscle stretched across the poles of the crescent. Male and female gametocytes are distinguished, designated respectively micro- and macro-gametocytes. As compared with the macro-gametocyte, the micro-gametocyte contains a nuclear structure richer in chromatin, and its pigment granules are finer. In the female gametocyte of *P. falciparum* the pigment is usually accumulated in the centre of the organism.

These gametocytes remain unchanged in the blood until it is withdrawn from the body—*e.g.* by the mosquito or when a drop is transferred to a warm stage for microscopic observation. In the stomach of the mosquito the following changes occur:—the gametocytes of the crescent type become rounded ; from

**BIOLOGICAL DIFFERENTIATION OF THE THREE
MALARIA ORGANISMS**

	P. VIVAX (<i>Benign tertian</i>)	P. MALARIAE (<i>Quartan</i>)	P. FALCIPARUM (<i>Malignant</i>)
Asexual life-cycle	48 hours	72 hours	Usually 48 hours
Fresh unstained preparation of blood . . .	<div style="display: inline-block; vertical-align: middle;"> <i>Amoebulae</i> </div> <div style="display: inline-block; vertical-align: middle; font-size: 3em; margin: 0 10px;">{</div> <div style="display: inline-block; vertical-align: middle;"> Not refractile, hyaline, not easily observed, usually single infections </div>	Refractile, "frosted-glass" appearance, more distinct	Very small, about $\frac{1}{16}$ th of diameter of R.B.C., often multiple infections
	<div style="display: inline-block; vertical-align: middle;"> <i>Movement</i> </div> <div style="display: inline-block; vertical-align: middle; font-size: 3em; margin: 0 10px;">}</div> <div style="display: inline-block; vertical-align: middle;">Active</div>	Not active	Very active at first
Pigment . . .	Fine, yellowish brown	Coarse, brownish black	Scanty, fine
Ring forms (stained preparations) .	Large, irregular, not well defined, usually single chromatin dot	Thick round rings, often in equatorial bands	Small, well defined, thin, often 2 chromatin dots, often flattened out on surface of R.B.C.
Red cell (stained preparations) .	Swollen, pale, showing "Schüffner's dots"	Not altered	Shrivalled, deeper colour, but may be swollen and pale
Schizont (stained preparations) .	Large, rosette-like, 15-20 oval merozoites	Small "daisy-head"-like, 7-10 round merozoites	Small, segmentation irregular, 8-25 very small merozoites, not seen in peripheral blood
Gametocytes .	Rounded	Rounded	Elongated, crescentic or sausage-shaped

the male cell flagella-like structures are quickly protruded ("exflagellation"); these are long slender processes with somewhat enlarged free ends, each containing a chromatin granule derived from the parent nucleus, and are the microgametes; they are ultimately detached, and move with a kind of lashing motility (exflagellation may be observed in fresh preparations of blood on a warm stage); the macrogametocyte undergoes "maturation"—*i.e.* the protrusion and detachment of one or two polar bodies containing part of the original nuclear chromatin—and becomes the macrogamete; a micro- and macrogamete unite and form the zygote or ookinete, which slightly elongates and penetrates the stomach wall, embedding itself between the muscle fibres; it rounds itself later, forms an encysting membrane ("sporocyst") and increases in size until it projects into the body cavity; division into rounded "sporoblasts" occurs, and these divide again into the spindle-shaped sporozoites; the cyst ultimately ruptures and the sporozoites are set free in the body cavity and settle in the veneno-salivary gland, from which they are injected with the salivary secretion when the insect bites; this phase in the life-history is that of sporogony as contrasted with the asexual schizogony in the human subject.

DIAGNOSIS

THIN BLOOD FILMS.—Two or three films are made on microscope slides or $\frac{3}{4}$ -in. square No. 1 cover-glasses which have been carefully cleansed (*vide* p. 93) and polished with a smooth cloth.

Apparatus required:—straight Hagedorn needle, slides or cover-glasses, spirit lamp or Bunsen, gauze, methylated spirit.

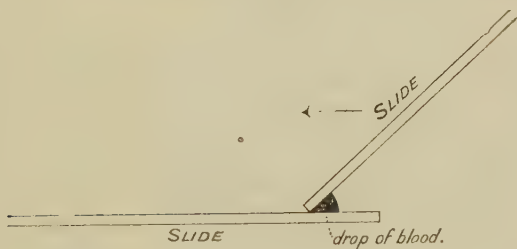
The blood is obtained by puncturing the lobe of the ear, or the finger close to the base of the nail. The needle and the area to be punctured are cleansed with spirit.

Films on slides.—Touch the exuding drop of blood

with the surface of a slide close to one end; then spread the drop of blood so removed over the whole length of the slide in the form of a thin uniform film; this is done by drawing the narrow edge of another slide, kept at an angle of 45° , slowly and gently along the first slide.

When the edge of the second slide touches the drop, allow the blood to spread out across the slide before drawing out the film.

Films on cover-glasses.—Touch the drop of blood with the surface of a cover-glass held by the edges



between the thumb and first finger of one hand, and place a second cover-glass over the first so that the drop spreads out between them. Then at once take the second glass by the edges between the thumb and forefinger of the other hand, and slide the two cover-glasses apart without exerting pressure.

The films are allowed to dry, and are then fixed and stained with Leishman's stain (*vide* p. 112). They are mounted and then examined, first with a dry $\frac{1}{6}$ -in. lens, and later with the oil-immersion. In searching for crescents it is advisable to use the former.

THICK BLOOD FILMS.—As a routine measure, and especially where the organisms are likely to be scanty, thick films should be prepared and examined. A large drop of blood is deposited on a slide, and spread with the head of a pin in the form of a *thick* film about $\frac{3}{4}$ in. in diameter; it is thoroughly dried—*e.g.* in the incubator—and the haemoglobin is removed by

treating with acid-alcohol (alcohol 50 c.c., hydrochloric acid 10 drops) and then washing in water; the film is stained by Leishman's method.

Note.—Films should, if possible, be taken during the pyrexia.

FRESH PREPARATIONS OF BLOOD FOR MICROSCOPIC EXAMINATION.—A drop of blood is deposited on a slide, covered with a cover-glass, and the edges of the glass are smeared with vaseline to prevent drying of the film. A warm-stage apparatus can be used during the microscopic examination.

Only stained films are examined as a rule in routine work. The organisms can be recognised by their various characteristic appearances, and it may be possible to determine the species or type present (*vide* Table, p. 269). It must be remembered however, that the young ring forms of the three types may be almost indistinguishable from one another, and if only young rings are present in the film, it may be difficult to determine the species. To inexperienced workers, artificial appearances may sometimes simulate malaria parasites, and a blood platelet overlying a red corpuscle may be mistaken for a young form of the plasmodium. In certain cases prolonged search may be required. At least two hundred microscope fields should be examined before the specimen is reported as negative. The absence of parasites during an apyrexial interval by no means excludes malaria. It is advisable, in searching for scanty malaria organisms, to examine particularly the edges of the film. Their frequency is usually greater at the edges than in the centre.

Certain *cytological features of the blood* may, in the absence of actual organisms, suggest the existence of a malarial infection—*e.g.* a relatively high percentage of large mononuclear cells (15–20 per cent.), or the presence of leucocytes containing altered blood pigment (*vide supra*).

TRYPANOSOMES PATHOGENIC TO MAN

Protozoa of the sub-class flagellata. Infection with these organisms is designated by the general term, Trypanosomiasis.

Three species are pathogenic to man: *T. gambiense* and *T. rhodesiense* of African Sleeping Sickness, and *T. cruzi* of Brazilian trypanosomiasis.

TRYPANOSOMA GAMBIENSE

Biology.—This organism is an elongated, sinuous, fusiform structure, $14-30\mu$ long by $1-3\mu$ broad, with a longitudinal undulating membrane and a flagellum projecting from one end. It is motile, and in moving the flagellum is anterior. In stained preparations two nuclear structures are noted, the larger or tropho-nucleus situated about the middle of the organism, and the smaller or kineto-nucleus at the posterior end. The latter stains deeply and is surrounded by an unstained halo. The flagellum seems to arise from the kineto-nucleus or its immediate vicinity, and forms the free edge of the undulating membrane before projecting anteriorly as a separate structure. The posterior end of the organism tends to be somewhat pointed.

Multiplication is by longitudinal amitotic fission.

Occurrence.—The infection is transmitted by the bite of *Glossina palpalis* (Tse-tse fly). It is uncertain whether the trypanosome undergoes a phase in its life-cycle in the fly. Shortly after blood from an infected person is ingested by the insect, transmission is possible in a mechanical fashion, but in a few days the trypanosomes disappear from the intestinal tract. In some cases however, a later stage of infectivity occurs (after about twenty days); the trypanosomes may multiply in the intestine, and pass to the proventriculus, the salivary glands and proboscis.

It is probable that *T. gambiense* occurs also in certain wild animals—*e.g.* the antelope—from which they are transmitted by tse-tse flies to man.

Two to three weeks after infection a febrile condition develops, and trypanosomes are present in the blood. The superficial lymph glands become enlarged, and trypanosomes can be demonstrated by puncture and aspiration with a syringe. In the advanced stages of the disease, when the characteristic lethargy has developed, the trypanosomes can be detected in the cerebro-spinal fluid.

Animal Inoculation.—Monkeys injected with infective material develop a disease which is more or less similar to human trypanosomiasis.

Guinea-pigs can be infected, and trypanosomes appear in considerable numbers in the blood, but the infection is either associated with no obvious pathological condition, or the resulting disease is very chronic in its course.

Cultivation of Trypanosomes.—See p. 75.

TRYPANOSOMA RHODESIENSE

Associated with a more acute form of Sleeping Sickness than that produced by *T. gambiense*.

Morphologically it resembles *T. gambiense*, but a certain number of the trypanosomes show the trophonucleus situated posteriorly near the kinetonucleus ("posterior nucleated" forms).

This species is also more virulent to laboratory animals.

It is transmitted by *Glossina morsitans*.

TRYPANOSOMA CRUZI

The cause of human trypanosomiasis in Brazil.

Its first development after infection occurs in the endothelial and tissue cells of internal organs, in the muscles, and in the heart wall. It is non-flagellate at first, and resembles a leishmania (*vide infra*). It may however, appear in the blood as a typical flagellate trypanosome.

It is transmitted by a bug (*Conorhinus megistus*).

Various laboratory animals are susceptible to experimental inoculation—*e.g.* guinea-pigs, white rats, monkeys.

DIAGNOSIS OF TRYPANOSOME INFECTION

In the first place the peripheral blood should be examined. As trypanosomes may be scanty, "thick-films" are prepared and stained by Leishman's stain as in malaria diagnosis (*q.v.*). Fresh preparations of the blood may also be examined microscopically.

A method of concentrating trypanosomes in the blood has been applied as follows:—5–10 c.c. of blood are withdrawn from a vein into 20 c.c. 1 per cent. sodium citrate solution, and the mixture is centrifugalised for about ten minutes; the plasma and leucocyte layer on the surface of the blood sediment are withdrawn and re-centrifugalised; this is repeated two or three times; the deposit is examined after each centrifugalisation.

If superficial lymph glands are enlarged, puncture and aspiration with a syringe may be carried out, and the "juice" examined. The syringe should be perfectly dry.

Blood, gland juice, or an emulsion of an excised gland injected into a guinea-pig may yield a positive diagnosis where other methods fail. The blood of the animal is examined in stained thin films at intervals after the inoculation.

In the lethargic state, 10 c.c. of cerebro-spinal fluid are withdrawn, and centrifugalised for fifteen to twenty minutes; the deposit is then examined either in the form of a fresh preparation under a cover-glass ringed with vaseline, or in stained films.

THE LEISHMANIAE

These are pathogenic protozoa biologically related to the trypanosomes, but more closely allied to the herpetomonas. Three separate species have been recognised:

L. donovani—of Kala-azar (occurring in certain Eastern countries).

L. tropica—of Tropical Ulcer or Delhi boil.

L. infantum—of Infantile Splenomegaly occurring in Northern Africa.

L. DONOVANI

Biology.—In morphology it is an oval organism about $2.5\text{--}3.5\mu$ in its longest diameter.

Stained with a Romanowsky stain two nuclear structures are observed, one large and rounded (macronucleus), and the other small, deeply staining, and rod-shaped (blepharoplast). The protoplasm may be vacuolated. The organism multiplies by binary fission.

The leishmaniae are typically intracellular in the tissues, situated in the endothelial cells of the spleen, liver, bone marrow and lymphatic glands. They may also be found in large mononuclear cells in the peripheral blood. One endothelial cell may contain considerable numbers of leishmaniae.

Cultures can be obtained from the spleen on N.N.N. medium (*vide* p. 75) incubated at $20^{\circ}\text{--}24^{\circ}\text{C}$. In culture the organisms increase in size and elongate; the blepharoplast becomes situated at one end, and from it a flagellum arises. No undulating membrane develops. Thus the leishmania in culture assumes the biological characters of a herpetomonas.

Monkeys and dogs can be infected experimentally.

Transmission of the disease is probably by insect agency. The responsible insect has not been definitely determined, though the bed-bug (*Cimex lectularius*) and the dog-flea (*Ctenocephalus canis*) have been suggested as possible vehicles of infection.

DIAGNOSIS

Thick blood films should be examined as in malaria diagnosis, but in a large proportion of cases it may be impossible to demonstrate leishmaniae in the peripheral blood.

During life the diagnosis may be established by aspirating fluid from the enlarged spleen by "spleen puncture." A dry needle should be used. This procedure is not without immediate danger to the patient, and for this reason liver puncture has been preferred by some workers, though the likelihood of finding the organism in fluid from the liver is less than in the case of the spleen.

It has also been claimed that a diagnosis may be established by culturing peripheral blood on N.N.N. medium.

L. TROPICA

This organism is similar to *L. donovani* and shows the same intracellular distribution. Besides the characteristic oval forms, elongated organisms may be noted. In culture on N.N.N. medium the herpetomonas forms develop as in the case of *L. donovani*. Monkeys and dogs can be infected experimentally. Transmission is probably by some insect—*e.g.* the bed-bug.

DIAGNOSIS

Diagnosis.—Films are made from the exudate of the ulcer after carefully cleansing the surface and removing the surface discharge. They are stained by Leishman's stain.

L. INFANTUM

This organism is similar to *L. donovani*, and is associated with a pathological condition, similar to that of Kala-azar, occurring in young children in North Africa and the Mediterranean littoral. The infection also occurs naturally in dogs, which are probably the origin of the human disease, the organism being transmitted by some insect—*e.g.* the dog-flea. Monkeys, dogs, guinea-pigs are susceptible to experimental infection.

Diagnosis is established as in the case of Kala-azar.

FILTERABLE VIRUSES

Considerable attention has been paid recently to a group of organisms which, either on account of their minute size or plasticity, are able to pass through the pores of earthenware filters.¹

Generally speaking, the filterable viruses are ultra-microscopic and have never been seen, although some—*e.g.* the virus of pleuropneumonia of cattle and *Bacterium pneumosintes*—are just within the range of visibility. Others—*e.g.* certain spirochaetes—are easily seen, but on account of their plasticity and motility are able to pass through some of the coarser filters.

It must be emphasised that “filterability” depends upon the size of the pores of the filter used; and whereas an organism might easily pass through a coarse filter and so be “filterable,” it may be held back by a finer one, and thus would be “non-filterable” with regard to that particular filter. Filterability may also depend on the pressure applied (*vide* p. 280).

When describing an organism as filterable, therefore, it is necessary to indicate (1) the type of filter used, and also (2) the pressure employed during filtration.

COARSE FILTERS

These are made from Kieselguhr, a diatomaceous earth found in deposits in Germany and other parts of the world. Filters made from this material are coarse—that is, have relatively large pores owing to the size of the granules forming the substance of the filter. It is through these filters only, that the visible organisms are able to pass. It has been computed that the size of the pores of a Berkefeld V filter is 0.2μ . As objects smaller than half the wave length of the light employed cannot be seen, the limit of visibility is about 0.1μ . It is thus obvious that organisms larger than 0.1μ but smaller than 0.2μ could be both filterable and visible. The commonest type of coarse filter is the

¹ Usually made in the form of cylindrical hollow “candles.”

Berkefeld, which is made in three grades of porosity—namely: V (viel) the coarsest, W (wenig) the finest, and N (normal) intermediate. Of these, the Berkefeld V is the one usually employed.

Berkefeld filters must always be tested before use, as they are particularly liable to imperfections, especially near the joint of the candle with the metal fitting. A Berkefeld V filter should not pass a small organism such as *B. melitensis* or *B. prodigiosus*. It is inadvisable sometimes to have the pores of the filter clogged by the test organisms, and the following method is very useful in detecting imperfections in manufacture. A source of compressed air, controlled by a pressure gauge, is connected to the filter, which is immersed in a glass beaker filled with water. The air is slowly turned on, and the pressure noted when air bubbles commence to escape. A candle suitable for filtration should maintain 8 to 12 lb. pressure, and when air bubbles do escape they should be uniform in size and density over the entire surface of the filter candle.

A similar type to the Berkefeld is the Mandler filter, manufactured in the United States. There is a British Berkefeld filter on the market, made in only one degree of porosity which is not specified by the makers.

FINE FILTERS

These are made of unglazed porcelain. The Chamberland (French) and Pukall (German) are examples. These will pass only certain ultramicroscopic viruses of extreme minuteness, such as the viruses of foot-and-mouth disease, of chicken plague and of hog cholera. The porcelain filters are used extensively for the removal of organisms from fluid cultures in order to obtain the bacterial toxin. Still finer filters may be made of collodion; these are of sufficient fineness to hold back even the virus of hog cholera, which passes porcelain filters of the smallest porosity. This indicates that the ultramicroscopic filterable viruses have each

definite sizes, and that they are not of the nature of a "*contagium fluidum*."

METHOD OF FILTRATION

It is advisable to filter with as small a pressure as practicable. Suction is the most satisfactory means, the fluid to be filtered being on the outside of the filter.

The following is one of the most convenient forms of *filtering apparatus*. A filter candle (Berkefeld type) is fitted by means of a screw and washers into a cylindrical glass mantle, and the metal tube of the filter passes through the rubber stopper of a conical filter flask of strong glass. This flask is provided with a lateral tube which can be connected with an exhaust pump by pressure tubing. The fluid is poured into the mantle and, after filtration, is collected in the flask. The necessary suction may be obtained by means of the usual form of water suction pump, or a Geryk air pump. An attached mercury manometer indicates the negative pressure.

By using a low pressure, the time of filtration is prolonged, but this may result in the passage of small motile flexible organisms such as spirochaetes and slender vibrios. When high pressure is used, small particles are rapidly forced into the pores of the candle, thus preventing further filtration. For ordinary purposes, a negative pressure of 200–300 mm. of mercury is sufficient.

When filtering small quantities of fluid, as in dealing with viruses, the Berkefeld candles, size $2\frac{1}{2} \times \frac{1}{2}$ in., are most convenient. In order to secure the maximum amount of filtrate, a test-tube slightly wider and longer than the candle, should be inverted over it. This ensures that the whole of the candle is covered with fluid almost to the end of filtration.

After use, the filters should be brushed with a stiff nail-brush in distilled water, and then boiled in distilled water. Before sterilising again, distilled

water should be run through them to show that they are pervious.

Before sterilisation, the glass mantle, the candle and test-tube covering are loosely assembled without tightening up the screw and washers. The open end of the glass cylinder is plugged with cotton-wool, and the metal tube of the candle is inserted into the rubber stopper of the filter flask. The whole is wrapped in "Kraft" brown paper and sterilised by steaming or autoclaving. It is advisable, where several filters and flasks are kept sterilised, to have corresponding numbers on the filters and flasks, to ensure the rubber stopper attached to the filter candle fitting into the correct flask.

When working with filterable viruses controls must always be made of the filtered material. It must be cultivated both aerobically and anaerobically to show that no ordinary bacteria are present. Inoculation of animals susceptible to the virus should be carried out, and the filtrate should give rise to the characteristic train of symptoms of the disease.

SOME SPECIFIC INFECTIOUS DISEASES OF MAN, ANIMALS AND PLANTS, THE AETIOLOGICAL AGENTS OF WHICH ARE ALL FILTERABLE AND, WITH FEW EXCEPTIONS, ULTRAMICROSCOPIC

Diseases of Man :

Yellow fever—visible—*Leptospira icteroides*.

Chicken-pox.

Smallpox.

Molluscum contagiosum.

Pappataci fever—visible—a *leptospira* has been reported as the aetiological agent.

Trachoma.

Rabies.

Measles.

Mumps.

Poliomyelitis—visible—"globoid bodies."

Influenza—visible—*Bacterium pneumosintes*.
Herpes febrilis and so-called encephalitis lethargica virus.

Common colds.

Dengue fever.

Diseases of Animals (only important ones indicated) :

Foot-and-mouth disease.

Pleuropneumonia of cattle.

African horse sickness—reported visible—a minute cocco-bacillus.

Sheep-pox and cow-pox.

Hog cholera.

Swamp fever of horses.

Distemper in dogs.

Rat and guinea-pig epizootics.

Cattle plague.

Catarrhal fever of sheep.

Infectious stomatitis papulosa of cattle.

Diseases of Birds, such as

Fowl pest.

Fowl diphtheria.

Leukaemia of fowls.

Pigeon-pox.

Diseases of Insects :

Silkworm jaundice.

Sac brood of bees.

Diseases of Plants :

Mosaic disease of tobacco and tomatoes.

GENERAL CHARACTER OF THE FILTERABLE VIRUSES

These infective agents are characterised by the following features, which are fairly constant for the whole series if we exclude the spirochaetes:—

1. Great infectiousness.
2. The production of active immunity, which is lasting.
3. Filterability.
4. Invisibility.
5. Non-cultivability.
6. Resistance to glycerol.
7. Wide geographical distribution.

1. GREAT INFECTIOUSNESS.—This is seen in small-pox epidemics, where, in spite of careful quarantine, cases continue to occur. Foot-and-mouth disease is another example; the difficulty of control is due to the amazing rapidity of spread, not only from animal to animal, but from one locality to another. Similarly the influenza epidemic exemplified the great infectiousness of a disease due to a filterable virus.

As a group, these viruses are markedly pathogenic, even in minute doses. The virus of febrile herpes is capable of causing infection of the rabbit with a quantity as small as 0.001 c.c. of a paper filtrate of a 10 per cent. emulsion of brain from an infected animal. Incredibly small amounts of the natural viruses are capable of causing infection, as is shown by the rapid spread of the disease in spite of the utmost attempts at control.

2. THE PRODUCTION OF ACTIVE IMMUNITY.—In individuals surviving infection, there is a high degree of immunity, which is usually permanent. Advantage is taken of this in immunisation against smallpox and rabies. In addition, specific antisera may be prepared, as in the case of hog cholera and cattle plague, which are available for specific treatment.

3. FILTERABILITY.—This aspect has been previously referred to and the term is merely a relative one.

4. INVISIBILITY.—Organisms smaller than 0.1μ are beyond the limits of visibility. A few, however, such as the virus of pleuropneumonia in cattle, *Bacterium pneumosintes* from influenza, are just on the limits of visibility, and their morphology can be studied. Viruses which can pass a Berkefeld W or a porcelain filter are too small to be seen with any system of microscope lenses.

5. NON-CULTIVABILITY.—The presence of the majority of these viruses is only indicated by their action on susceptible animals. The infective agents are so highly parasitic that, with few exceptions, they have not been cultivated outside the body on any artificial media, in spite of the vast amount of work done on this subject. Several have, however, been transmitted through many generations without animal

passage. By means of the Smith-Noguchi medium, the "globoid bodies" of poliomyelitis, the *Bacterium pneumosintes* of epidemic influenza have been cultivated. Nocard and Roux succeeded in growing the virus of pleuro-pneumonia of cattle in a collodion sac inside the animal body, while Marchoux has propagated the aetiological agent of fowl pest through several generations on chicken-blood glucose agar. With these exceptions, the filterable viruses (excluding spirochaetes) are non-cultivable.

6. **RESISTANCE TO GLYCEROL.**—Ordinary bacteria are killed by 50 per cent. glycerin in a comparatively short time. Many of the filterable viruses, on the contrary, retain their infectivity much longer in this material than in any other fluid when kept at 4° C. The preservation of the vaccine virus used prophylactically against smallpox is accomplished by this means. Other viruses which keep for prolonged periods in glycerol at 4° C. are: poliomyelitis virus, the virus of epidemic influenza, the virus of febrile herpes (which we have kept virulent in glycerin for more than one year), and the virus of rabies. It is essential that the glycerol be pure and free from mineral acids and sulphates.

The filterable viruses are very readily destroyed by heat and antiseptics, but may survive outside the body for considerable periods of time.

7. **WIDE GEOGRAPHICAL DISTRIBUTION.**—The majority of the filterable viruses cause diseases which are not limited during periods of epidemic prevalence to any one geographical area. Measles, smallpox, influenza, foot-and-mouth disease, are universally distributed. An outbreak in one country is liable to be rapidly followed by outbreaks in surrounding countries, and even in other continents.

BACTERIUM PNEUMOSINTES

(Olitsky and Gates)

Morphology and Staining.—This is a minute coccobacillary organism measuring 0.15–0.2 μ in length.

After prolonged artificial culture, its length may attain 0.5μ . The organisms are usually found singly, but may be in diplo-form, and even in short chains of three or four individuals. Bacterium pneumosintes is stained best with well-ripened polychrome methylene blue, when the organisms appear deep purple in colour. It is Gram-negative.

Filterability.—Passes Berkefeld V and N filters.

Culture.—Obligatory anaerobe. Optimum temperature— 37.5°C . Grows well in Smith-Noguchi medium (p. 73), forming a haze in the medium around the fragment of kidney in three to four days. Cultures will remain viable for several months in the ice-box. On anaerobic blood-agar plates minute clear transparent colonies are formed after about six days incubation.

Resistance.—Remains viable in 50 per cent. glycerin at 4°C . for several months.

Experimental Inoculation.—Inoculated intra-tracheally into rabbits it produces fever, marked leucopenia due to a diminution of mononuclear cells, and haemorrhages in the lung. It is not fatal for these animals, but owing to a lowering of resistance, the rabbit may succumb to a secondary infection.

Occurrence.—Found in the nasal washings of epidemic influenza in the first thirty-six hours of the disease. It has never been found in healthy persons nor in other inflammatory conditions of the respiratory tract.

This organism is regarded by some authorities as the probable aetiological agent in epidemic influenza.

THE POLIOMYELITIS VIRUS

Definition.—An acute infection occurring chiefly in children, which affects the central nervous system, with usually a special localisation in the anterior horn of the spinal cord.

Filterability.—The virus can pass through Berkefeld V and N filters.

Cultivation.—Flexner and Noguchi, using Smith-Noguchi medium, have cultivated minute bodies

which are probably the infective agent. Using human brain tissue from a fatal case for inoculation, cloudiness was noted in the medium round the kidney tissue after five to seven days incubation.

Morphology.—In films from the culture stained by Giemsa's method, minute "globoid bodies," about 0.2μ in diameter and stained purple in colour, are noted. They occur in pairs, in short chains, and occasionally in small clusters. The "globoid bodies" are usually Gram-negative.

Resistance.—The virus is easily destroyed by heat, but can withstand the action of weak phenol for some days. In brain tissue, preserved in 50 per cent. glycerol in the ice-box, the virus remains viable for years.

Experimental Inoculation.—The disease can be transmitted to monkeys by the intracerebral or intravenous inoculation of human brain or spinal cord. The animals develop the typical disease, and show histological lesions in the central nervous system similar to human cases. The disease can also be transmitted from monkey to monkey.

Inoculation of monkeys with cultures of "globoid bodies" also reproduces the disease in these animals.

Occurrence.—Infection probably takes place through the nasal mucous membranes to the olfactory lobes of the brain. The disease is spread by carriers, who harbour the virus in the naso-pharynx.

RABIES

This disease is communicated to the human subject by the bite of a rabid dog, the infective virus being present in the animal's saliva. When a person is bitten by a dog suffering from rabies, prophylactic treatment by the Pasteur method must be carried out without delay, and where there is any doubt as to the condition of the dog, an accurate diagnosis is essential. The animal should be killed, and, if the laboratory is at some distance, the head is removed and forwarded in ice. In the laboratory the scalp is reflected, the skull is opened by means of sterile bone forceps and the brain

removed with aseptic precautions. The hippocampus, which forms the floor of the lateral ventricle, is dissected out, smears are made by squeezing a portion of the tissue between two slides, and pieces are also fixed for histological examination. In addition, an emulsion is prepared for animal inoculation. The smears are fixed in methyl alcohol for five minutes, and stained by Giemsa's or Leishman's method (*vide* pp. 112, 113). For sections, the tissue is fixed in formalin (*vide* p. 119) and stained as above.

The diagnosis depends on the finding of the characteristic Negri bodies in the cytoplasm of the nerve cells. They consist of round, oval or angular bodies, varying in size from $0.5-25\mu$, staining pink with Leishman's or Giemsa's stain. The nature of these structures is still a matter of uncertainty, though their occurrence is a specific feature of rabies.

Animal inoculation is carried out as follows:—

A portion of the brain tissue is ground up in a sterile mortar with sterile saline solution, and a rabbit is inoculated intracerebrally with 0.25 c.c. of the emulsion. The head of the rabbit is shaved and sterilised with tincture of iodine. The animal is then anaesthetised with ether, and an incision is made in the skin midway between one eye and the ridge in the middle line at the junction of the parietal and occipital bones. The skull is pierced with a small trephine and the material is injected, using a short ($\frac{1}{4}$ -in.) needle fitted to a 1 c.c. syringe. If the rabies virus is present, the animal becomes paretic in from seven to twenty-three days, and death occurs in fifteen to twenty-eight days. Negri bodies can also be demonstrated in smears or sections from the brain.

THE LABORATORY INVESTIGATION OF TYPHUS FEVER

This disease is transmitted by the body louse. The virus can be communicated to guinea-pigs by the intraperitoneal injection of 3-5 c.c. of patient's

blood taken during the height of the illness. After an incubation period of about nine days, the animal's temperature rises to 105° F., and remains above the normal for four to eight days. The guinea-pig does not succumb to experimental typhus infection, and on recovery does not react to a second injection of virus. If the animal is killed on the first or second day of the fever, the heart blood is infective for other guinea-pigs, but does not contain any recognisable bacteria. At autopsy, the spleen is enlarged and the peritoneum covering it is dull in appearance. The skin, on microscopic examination, shows the presence of an exanthematous reaction.

The virus is present in the blood, spleen, brain and other organs. It has never been obtained apart from tissue. It is easily destroyed by heat and antiseptics. It has not been cultivated and is not filterable.

Some observers regard the aetiological agent of typhus fever to be minute oval or bacilloid bodies staining by Giemsa's method, and termed *Rickettsia prowazeki*. Similar bodies can be demonstrated in smears from infected lice.

The *B. proteus* strain originally found in the urine of typhus patients, and used for the Weil-Felix reaction, has no aetiological relationship to the disease.

DIAGNOSIS

The Weil-Felix reaction is performed in exactly the same manner as the Widal test (p. 136), except that an emulsion of *B. proteus* ("X 19") is used instead of a member of the enteric group. The minimum titre acceptable for diagnostic purposes is 1 in 100, but agglutination frequently occurs in much higher dilutions—*e.g.* 1 in 1500.

Inoculation of the guinea-pig may also be carried out (*vide supra*).

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